PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B4

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/32, A01N 63/00
C12N 15/82, 1/21, C12P 1/04

A2

(11) International Publication Number: WO 93/15206

(43) International Publication Date: 5 August 1993 (05.08.93)

(21) International Application Number: PCT/US93/00966

(22) International Filing Date: 1 February 1993 (01.02.93)

(30) Priority data:
07/828,430 30 January 1992 (30.01.92) US

(71) Applicant: MYCOGEN CORPORATION [US/US]; 4980 Carroll Canyon Road, San Diego, CA 92121 (US).

(72) Inventors: MICHAELS, Tracy, E. ; 1110 Fern Street, Escondido, CA 92027 (US). FONCERRADA, Louis ; 322 Ferrara Way, Vista, CA 92083 (US). NARVA, Kenneth, E. ; 12123 Caminito Mira Del Mar, San Diego, CA 92130 (US).

(74) Agents: SALIWANCHIK, David, R. et al.; Saliwanchik & Saliwanchik, 2421 N.W. 41st Street, Suite A-1, Gainesville, FL 32606 (US).

(81) Designated States: AU, BR, CA, JP, KR, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: PROCESS FOR CONTROLLING SCARAB PESTS WITH *BACILLUS THURINGIENSIS* ISOLATES

(57) Abstract

Certain isolates of *Bacillus thuringiensis* (B.t.) have been found to have activity against scarab pests. These isolates are designated B.t. PS86B1, B.t. PS43F and B.t. PS50C. These isolates, or transformed hosts containing the gene expressing a scarab-active toxin obtained from the isolates, can be used to control scarab-active pests, e.g., masked chafer, *Cyclocephala* sp., June bettle, *Cotinis* sp., northern masked chafer, *Cyclocephala borealis*, Japanese beetle, *Popillia japonica*, and Pasadena masked chafer, *Cyclocephala pasadenae*, in various environments.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

DESCRIPTION

PROCESS FOR CONTROLLING SCARAB PESTS WITH *BACILLUS THURINGIENSIS* ISOLATES

Cross-Reference to a Related Application

This is a continuation-in-part of co-pending application Serial No. 07/828,430, filed January 30, 1992, which is a continuation-in-part of co-pending application Serial No. 07/808,316, filed on December 16, 1991, now abandoned.

Background of the Invention

The soil microbe *Bacillus thuringiensis* (*B.t.*) is a Gram-positive, spore-forming bacterium characterized by parasporal crystalline protein inclusions. These often appear microscopically as distinctively shaped crystals. The proteins can be highly toxic to pests and specific in their activity. Certain *B.t.* toxin genes have been isolated and sequenced, and recombinant DNA-based *B.t.* products produced and approved. In addition, with the use of genetic engineering techniques, new approaches for delivering *B.t.* endotoxins to agricultural environments are under development, including the use of plants genetically engineered with endotoxin genes for insect resistance and the use of stabilized intact microbial cells as *B.t.* endotoxin delivery vehicles (Gaertner, F.H., L. Kim [1988] *TIBTECH* 6:S4-S7). Thus, isolated *B.t.* endotoxin genes are becoming commercially valuable.

Over the past 30 years, commercial use of *B.t.* pesticides has been largely restricted to a narrow range of lepidopteran (caterpillar) pests. Preparations of the spores and crystals of *B. thuringiensis* subsp. *kurstaki* have been used for many years as commercial insecticides for lepidopteran pests. For example, *B. thuringiensis* var. *kurstaki* HD-1 produces a crystal called a delta endotoxin which is toxic to the larvae of a number of lepidopteran insects.

In recent years, however, investigators have discovered *B.t.* pesticides with specificities for a much broader range of pests. For example, other species of *B.t.*, namely *israelensis* and *san diego* (a.k.a. *B.t. tenebrionis*, a.k.a. M-7), have been used commercially to control insects of the orders Diptera and Coleoptera, respectively

(Gaertner, F.H. [1989] "Cellular Delivery Systems for Insecticidal Proteins: Living and Non-Living Microorganisms," in *Controlled Delivery of Crop Protection Agents*, R.M. Wilkins, ed., Taylor and Francis, New York and London, 1990, pp. 245-255). See also Couch, T.L. (1980) "Mosquito Pathogenicity of *Bacillus thuringiensis* var. *israelensis*," *Developments in Industrial Microbiology* 22:61-76; Beegle, C.C., (1978) "Use of Entomogenous Bacteria in Agroecosystems," *Developments in Industrial Microbiology* 20:97-104. Krieg, A., A.M. Huger, G.A. Langenbruch, W. Schnetter (1983) *Z. ang. Ent.* 96:500-508, describe a *B.t.* isolate named *Bacillus thuringiensis* var. *tenebrionis*, which is reportedly active against two beetles in the order Coleoptera. These are the Colorado potato beetle, *Leptinotarsa decemlineata*, and *Agelastica alni*.

Recently, new subspecies of *B.t.* have been identified, and genes responsible for active δ-endotoxin proteins have been isolated (Höfte, H., H.R. Whiteley [1989] *Microbiological Reviews* 52(2):242-255). Höfte and Whiteley classified *B.t.* crystal protein genes into 4 major classes. The classes were CryI (Lepidoptera-specific), CryII (Lepidoptera- and Diptera-specific), CryIII (Coleoptera-specific), and CryIV (Diptera-specific). The discovery of strains specifically toxic to other pests has been reported. (Feitelson, J.S., J. Payne, L. Kim [1992] *Bio/Technology* 10:271-275).

The cloning and expression of a *B.t.* crystal protein gene in *Escherichia coli* has been described in the published literature (Schnepf, H.E., H.R. Whitely [1981] *Proc. Natl. Acad. Sci. USA* 78:2893-2897). U.S. Patent 4,448,885 and U.S. Patent 4,467,036 both disclose the expression of *B.t.* crystal protein in *E. coli*. U.S. Patents 4,797,276 and 4,853,331 disclose *B. thuringiensis* strain *san diego* (a.k.a. *B.t. tenebrionis*, a.k.a. M-7) which can be used to control coleopteran pests in various environments. U.S. Patent No. 5,151,363 discloses certain isolates of *B.t.* which have activity against nematodes. Many other patents have issued for new *B.t.* isolates and new uses of *B.t.* isolates. The discovery of new *B.t.* isolates and new uses of known *B.t.* isolates remains an empirical, unpredictable art.

Insects in the family Scarabaeidae (scarabs) constitute a serious pest control problem, especially when destructive larval stage insects infest high value turf found in golf courses, playing fields and lawns. The larvae of many species also attack grains, tuberous crops, and ornamentals. Larvae are called "white grubs" or "chafer grubs" and can be found in decaying organic matter (rotting leaves, manure, etc.) or

2-10 cm. deep in soil where they consume the plant roots. In turf infested areas there can be as many as 30 grubs per square foot. The damage caused by an infestation becomes most apparent in the fall when the third instar grubs are feeding. Adult beetles of some scarab species will feed on a wide variety of vegetative host, damaging foliage, fruit and flowers of woody and herbaceous plants. In the U.S. and Europe, populations of larvae and adults have developed resistance to chemical insecticides such as the organochlorines and DDT.

Several scarab pests are of economic importance. Particularly important pests in the U.S., especially east of the Rockies, but also in the Western States, are the masked chafers, *Cyclocephala* sp. In the east, the northern masked chafer, *C. borealis*, and the southern masked chafer, *C. immaculata*, are common pests, while, in California, *C. hirta* and *C. pasadenae* are present. Also, in the U.S., especially in the area east of the rockies, infestations of Japanese beetles *Popillia* sp., May or June beetles *Phyllophaga* sp., black turfgrass beetles *Ataenius* sp., European chafers *Rhizotrogus* sp., tend to necessitate the greatest amount of insecticide treatments. Other important scarab pests in the U.S. can be quite damaging but localized such as with Oriental beetles *Anomala* sp., hoplia chafers *Hoplia* sp., green June beetle *Cotinis* sp., and Asiatic garden beetles *Maladera* sp. Several scarabs not present in the U.S. are of economic importance in Europe, including rose chafers *Cetonia* sp., cockchafers *Melolontha* sp., flower beetles *Adoretus* sp., and garden chafers *Phyllopertha* sp. The green June beetles, *Cotinis* sp., can cause serious damage where populations become abundant. The adults are attracted to ripening fruit and will devour figs, peaches and other thin skinned fruit while on the tree. Larvae are attracted to decaying organic matter and most commonly become pests in turf or fields which have been fertilized with manure. The feeding and tunnelling of the large larvae can become disruptive. The eastern green June beetle *Cotinis nitida* is present in the mid-western and eastern states, while the green June beetle *C. mutabilis* occurs in many of the western states.

Brief Summary of the Invention

The subject invention concerns novel materials and methods for controlling scarab pests. The materials and methods of the subject invention result from the unexpected discovery that certain *B.t.* isolates have activity against these pests.

More specifically, the methods of the subject invention use *B.t.* microbes, or variants thereof, and/or their toxins, to control scarab pests. Specific *B.t.* microbes useful according to the invention are *B.t.* PS86B1, *B.t.* PS43F, and *B.t.* PS50C. Further, the subject invention also includes the use of variants of the exemplified *B.t.* isolates which have substantially the same scarab-active properties as the specifically exemplified *B.t.* isolates. Such variants would include, for example, mutants. Procedures for making mutants are well known in the microbiological art. Ultraviolet light and nitrosoguanidine are used extensively toward this end.

The subject invention also includes the use of genes from the *B.t.* isolates of the invention which genes encode the scarab-active toxins.

Still further, the invention also includes the treatment of substantially intact *B.t.* cells, or recombinant cells containing the genes of the invention, to prolong the scarab activity when the substantially intact cells are applied to the environment of a target pest. Such treatment can be by chemical or physical means, or a combination of chemical and physical means, so long as the technique does not deleteriously affect the properties of the pesticide, nor diminish the cellular capability in protecting the pesticide. The treated cell acts as a protective coating for the pesticidal toxin. The toxin becomes available to act as such upon ingestion by a target insect.

Finally, the subject invention further concerns plants which have been transformed with genes encoding scarab-active toxins.

Brief Description of the Sequences

SEQ ID NO. 1 is the nucleotide sequence (open reading frame only) of the gene designated 50C(a).

SEQ ID NO. 2 is the predicted amino acid sequence of the toxin 50C(a).

SEQ ID NO. 3 is the nucleotide sequence (open reading frame only) of the gene designated 50C(b).

SEQ ID NO. 4 is the predicted amino acid sequence of the toxin 50C(b).

SEQ ID NO. 5 is the composite nucleotide sequence and deduced amino acid sequence of the gene designated 43F.

SEQ ID NO. 6 is the predicted amino acid sequence of the toxin 43F.

Detailed Disclosure of the Invention

The subject invention concerns the use of selected strains of *Bacillus thuringiensis* for the control of scarab pests.

Specific *Bacillus thuringiensis* isolates useful according to the subject invention have the following characteristics in their biologically pure form:

Characteristics of *B.t.* PS50C

Colony morphology--Large colony, dull surface, typical *B.t.*

Vegetative cell morphology--typical *B.t.*

Culture methods--typical for *B.t.*

Flagellar serotyping--PS50C belongs to serotype 18, kumamotoensis.

Crystal morphology--a sphere.

RFLP analysis--Southern hybridization of total DNA distinguishes *B.t.* PS50C from *B.t.s.d.* and other *B.t.* isolates.

Alkali-soluble proteins--SDS polyacrylamide gel electrophoresis (SDS-PAGE) shows a 130 kDa doublet protein.

A comparison of the characteristics of *B. thuringiensis* PS50C (*B.t.* PS50C) to the characteristics of the known *B.t.* strains *B. thuringiensis* var. *san diego* (*B.t.s.d.*), *B. thuringiensis* PS86B1 (NRRL B-18299), and *B. thuringiensis* var. *kurstaki* (HD-1) is shown in Table 1.

Table 1. Comparison of *B.t.* PS50C, *B.t.* PS86B1, *B.t.s.d.*, and *B.t.* HD-1

	B.t. PS50C	*B.t.s.d.*	*B.t.* PS86B1	*B.t.* HD-1
Serovar	kumamotoensis	morrisoni	tolworthi	kurstaki
Type of inclusion	sphere	square wafer	flat, pointed ellipse, plus sm. inclusions	Bipyramid
Size of alkali-soluble proteins by SDS-PAGE	130 kDa doublet	72,000 64,000	75,000 68,000 61,000	130,000 68,000
Host range	Coleoptera	Coleoptera	Coleoptera	Lepidoptera

B.t. isolates useful according to the subject invention have been deposited. Also deposited are recombinant microbes comprising the *B.t.* genes of interest.

Culture	Accession Number	Deposit Date
Bacillus thuringiensis PS86B1	NRRL B-18299	February 3, 1988
Bacillus thuringiensis PS43F	NRRL B-18298	February 3, 1988
E. coli XL1-Blue(pM1,98-4)	NRRL B-18291	January 15, 1988
Bacillus thuringiensis PS50C	NRRL B-18746	January 9, 1991
E. coli NM522(pMYC1638)	NRRL B-18751	January 11, 1991

The cultures are on deposit in the permanent collection of the Northern Research Laboratory, U.S. Department of Agriculture, Peoria, IL, USA.

The subject cultures have been deposited under conditions that assure that access to the cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of

Microorganisms, i.e., they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposit, and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the cultures. The depositor acknowledges the duty to replace the deposits should the depository be unable to furnish a sample when requested, due to the condition of the deposit(s). All restrictions on the availability to the public of the subject culture deposits will be irrevocably removed upon the granting of a patent disclosing them.

Genes and toxins. The genes and toxins according to the subject invention include not only the full length sequences disclosed herein but also fragments of these sequences, or fusion proteins, which retain the characteristic pesticidal activity of the toxins specifically exemplified herein.

It should be apparent to a person skilled in this art that genes coding for scarab-active toxins can be identified and obtained through several means. The specific genes exemplified herein may be obtained from the isolates deposited at a culture depository as described above. These genes, or portions or variants thereof, may also be constructed synthetically, for example, by use of a gene machine. As used herein, the terms "variants" or "variations" of genes refer to nucleotide sequences which code for the same toxins or which code for equivalent toxins having scarab activity. Variations of these genes may be readily constructed using standard techniques for making point mutations. Also, fragments of these genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes which code for active fragments may be obtained using a variety of other restriction enzymes. Proteases may be used to directly obtain active fragments of these toxins.

Equivalent toxins and/or genes encoding these equivalent toxins can also be located from *B.t.* isolates and/or DNA libraries using the teachings provided herein. These "equivalent" toxins and genes are also referred to herein as "variant" toxins or genes. There are a number of methods for obtaining the pesticidal toxins of the instant invention. For example, antibodies to the pesticidal toxins disclosed and

claimed herein can be used to identify and isolate other toxins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the toxins which are most constant and most distinct from other *B.t.* toxins. These antibodies can then be used to specifically identify equivalent toxins with the characteristic activity by immunoprecipitation, enzyme linked immunoassay (ELISA), or Western blotting. Antibodies to the toxins disclosed herein, or to equivalent toxins, or fragments of these toxins, can readily be prepared using standard procedures in this art. The genes coding for these toxins can then be obtained from the microorganism.

A further method for identifying the toxins and genes of the subject invention is through the use of oligonucleotide probes. These probes are nucleotide sequences having a detectable label. As is well known in the art, if the probe molecule and nucleic acid sample hybridize by forming a strong bond between the two molecules, it can be reasonably assumed that the probe and sample have substantial homology. The probe's detectable label provides a means for determining in a known manner whether hybridization has occurred. Such a probe analysis provides a rapid method for identifying toxin-encoding genes of the subject invention. The nucleotide segments which are used as probes according to the invention can be synthesized by use of DNA synthesizers using standard procedures.

Fragments and mutations of the exemplified toxins, which retain the pesticidal activity of the exemplified toxins, would be within the scope of the subject invention, as would genes which encode such fragments and mutants. Also, because of the redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create these alternative DNA sequences encoding the same, or essentially the same, toxins. These variant DNA sequences are within the scope of the subject invention. As used herein, reference to "essentially the same" sequence refers to sequences which have amino acid substitutions, deletions, additions, or insertions which do not materially affect pesticidal activity. Fragments retaining scarab activity are also included in this definition. As used herein, the phrase "scarab activity" includes activity against scarab larvae as well as other stages of development.

Toxins of the subject invention are specifically exemplified herein by the toxins encoded by the genes designated 50C(a), 50C(b), and 43F. Since these toxins are

merely exemplary of the toxins of the subject invention, it should be readily apparent that the subject invention further comprises variant toxins (and nucleotide sequences coding for variant toxins) having the same or essentially the same biological activity against scarab pests of the exemplified toxins. These equivalent toxins will have amino acid homology with a toxin of the subject invention. This amino acid homology will typically be greater than 75%, preferably be greater than 90%, and most preferably be greater than 95%. The amino acid homology will be highest in certain critical regions of the toxins which account for biological activity or are involved in the determination of three-dimensional configuration which ultimately is responsible for the biological activity. In this regard, certain amino acid substitutions are acceptable and can be expected if these substitutions are in regions which are not critical to activity or are conservative amino acid substitutions which do not affect the three-dimensional configuration of the molecule. For example, amino acids may be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution does not materially alter the biological activity of the compound. Table 2 provides a listing of examples of amino acids belonging to each class.

Table 2

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

In some instances, non-conservative substitutions can also be made. The critical factor is that these substitutions must not significantly detract from the biological activity of the toxin.

The toxins of the subject invention can also be characterized in terms of the shape and location of toxin inclusions, which are described above.

Recombinant hosts. The toxin-encoding genes harbored by the isolates of the subject invention can be introduced into a wide variety of microbial or plant hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. With suitable microbial hosts, e.g., *Pseudomonas*, the microbes can be applied to the situs of scarab pests where they will proliferate and be ingested by the pest. The result is a control of this pest. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin produced in the cell. The treated cell then can be applied to the environment of the target pest. The resulting product retains the toxicity of the *B.t.* toxin.

Where the *B.t.* toxin gene is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is essential that certain host microbes be used. Microorganism hosts are selected which are known to occupy the soil. These microorganisms are selected so as to be capable of successfully competing in the soil with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably, provide for improved protection of the pesticide from environmental degradation and inactivation.

A large number of microorganisms are known to inhabit the rhizosphere (the soil surrounding plant roots). These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera *Bacillus*, *Pseudomonas*, *Erwinia*, *Serratia*, *Klebsiella*, *Xanthomonas*, *Streptomyces*, *Rhizobium*, *Rhodopseudomonas*, *Methylophilius*, *Agrobacterium*, *Acetobacter*, *Lactobacillus*, *Arthrobacter*, *Azotobacter*, *Leuconostoc*, *Alcaligenes* and *Clostridium*; fungi, particularly yeast, e.g., genera *Saccharomyces*, *Cryptococcus*, *Kluyveromyces*, *Sporobolomyces*, *Rhodotorula*, and *Aureobasidium*; microalgae, e.g., families *Cyanophyceae*, *Prochlorophyceae*, *Rhodophyceae*, *Dinophyceae*, *Chrysophyceae*, *Prymnesiophyceae*, *Xanthophyceae*, *Raphidophyceae*, *Bacillariophyceae*, *Eustigmatophyceae*, *Cryptophyceae*, *Euglenophyceae*, *Prasinophyceae*, and *Chlorophyceae*. Of particular interest are such phytosphere bacterial species as

Pseudomonas syringae. Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacterium tumefaciens, Rhodopseudomonas spheroides, Xanthomonas campestris, Rhizobium melioti, Alcaligenes entrophus, and *Azotobacter vinlandii*; and phytosphere yeast species such as *Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus, C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces roseus, S. odorus, Kluyveromyces veronae,* and *Aureobasidium pollulans.* Of particular interest are the pigmented microorganisms.

A wide variety of ways are available for introducing a *B.t.* gene encoding a toxin into a microorganism host under conditions which allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in United States Patent No. 5,135,867, which is incorporated herein by reference.

Treatment of cells. As mentioned above, *B.t.* or recombinant cells expressing a *B.t.* toxin can be treated to prolong activity in the environment. Suitable host cells, where the pesticide-containing cells will be treated to prolong the activity of the toxin in the cell when the then treated cell is applied to the environment of target pest(s), may include either prokaryotes or eukaryotes, normally being limited to those cells which do not produce substances toxic to higher organisms, such as mammals. However, organisms which produce substances toxic to higher organisms could be used, where the toxin is unstable or the level of application sufficiently low as to avoid any possibility of toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi.

The cell will usually be intact and be substantially in the proliferative form when treated, rather than in a spore form, although in some instances spores may be employed.

Treatment of the microbial cell, e.g., a microbe containing the *B.t.* toxin gene, can be by chemical or physical means, or by a combination of chemical and/or physical means, so long as the technique does not deleteriously affect the properties of the toxin, nor diminish the cellular capability in protecting the toxin. Examples of chemical reagents are halogenating agents, particularly halogens of atomic no. 17-80. More particularly, iodine can be used under mild conditions and for sufficient time to

achieve the desired results. Other suitable techniques include treatment with aldehydes, such as formaldehyde and glutaraldehyde; anti-infectives, such as zephiran chloride and cetylpyridinium chloride; alcohols, such as isopropyl and ethanol; various histologic fixatives, such as Lugol iodine, Bouin's fixative, and Helly's fixative (See: Humason, Gretchen L., *Animal Tissue Techniques*, W.H. Freeman and Company, 1967); or a combination of physical (heat) and chemical agents that preserve and prolong the activity of the toxin produced in the cell when the cell is administered to the host animal. Examples of physical means are short wavelength radiation such as gamma-radiation and X-radiation, freezing, UV irradiation, lyophilization, and the like.

The cells generally will have enhanced structural stability which will enhance resistance to environmental conditions. Where the pesticide is in a proform, the method of inactivation should be selected so as not to inhibit processing of the proform to the mature form of the pesticide by the target pest pathogen. For example, formaldehyde will crosslink proteins and could inhibit processing of the proform of a polypeptide pesticide. The method of inactivation or killing retains at least a substantial portion of the bio-availability or bioactivity of the toxin.

Characteristics of particular interest in selecting a host cell for purposes of production include ease of introducing the *B.t.* gene into the host, availability of expression systems, efficiency of expression, stability of the pesticide in the host, and the presence of auxiliary genetic capabilities. Characteristics of interest for use as a pesticide microcapsule include protective qualities for the pesticide, such as thick cell walls, pigmentation, and intracellular packaging or formation of inclusion bodies; survival in aqueous environments; lack of mammalian toxicity; attractiveness to pests for ingestion; ease of killing and fixing without damage to the toxin; and the like. Other considerations include ease of formulation and handling, economics, storage stability, and the like.

Growth of cells. The cellular host containing the *B.t.* insecticidal gene may be grown in any convenient nutrient medium, where the DNA construct provides a selective advantage, providing for a selective medium so that substantially all or all of the cells retain the *B.t.* gene. These cells may then be harvested in accordance with conventional ways. Alternatively, the cells can be treated prior to harvesting.

The *B.t.* cells of the invention can be cultured using standard art media and fermentation techniques. Upon completion of the fermentation cycle the bacteria can be harvested by first separating the *B.t.* spores and crystals from the fermentation broth by means well known in the art. The recovered *B.t.* spores and crystals can be formulated into a wettable powder, liquid concentrate, granules or other formulations by the addition of surfactants, dispersants, inert carriers, and other components to facilitate handling and application for particular target pests. These formulations and application procedures are all well known in the art.

Formulations. Formulated bait granules containing an attractant and spores and crystals of the *B.t.* isolates, or recombinant microbes comprising the gene(s) obtainable from the *B.t.* isolates disclosed herein, can be applied to the soil. Formulated product can also be applied as a seed-coating or root treatment or total plant treatment at later stages of the crop cycle.

As would be appreciated by a person skilled in the art, the pesticidal concentration will vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly. The pesticide will be present in at least 1% by weight and may be 100% by weight. The dry formulations will have from about 1-95% by weight of the pesticide while the liquid formulations will generally be from about 1-60% by weight of the solids in the liquid phase. The formulations will generally have from about 10^2 to about 10^4 cells/mg. These formulations will be administered at about 50 mg (liquid or dry) to 1 kg or more per hectare.

The formulations can be applied to the environment of the scarab, e.g., soil, by spraying, dusting, sprinkling, or the like.

The *B.t.* pesticide of the invention can be applied to the soil to control scarab larvae as follows:

— a granule to the soil

— a granule mixed with sand, which fills holes during aeration of turf

— a granule with a sub-surface applicator upon re-seeding in turf

— a spray to the soil (soil drench)

— a spray following aeration

— a spray applied with sub-surface applicator

— combined with a water holding polymer placed in soil with a sub-surface applicator.

B.t. pesticidal treatment for adult scarab pests can be done as follows:

— granules with attractant, dispersed in area where beetles are flying

— attractant bait where beetles can congregate to feed

— as a foliar spray to host plant.

Mutants. Mutants of the novel isolates of the invention can be made by procedures well known in the art. For example, an asporogenous mutant can be obtained through ethylmethane sulfonate (EMS) mutagenesis of a novel isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

A smaller percentage of the asporogenous mutants will remain intact and not lyse for extended fermentation periods; these strains are designated lysis minus (—). Lysis minus strains can be identified by screening asporogenous mutants in shake flask media and selecting those mutants that are still intact and contain toxin crystals at the end of the fermentation. Lysis minus strains are suitable for a cell fixation process that will yield a protected, encapsulated toxin protein.

To prepare a phage resistant variant of said asporogenous mutant, an aliquot of the phage lysate is spread onto nutrient agar and allowed to dry. An aliquot of the phage sensitive bacterial strain is then plated directly over the dried lysate and allowed to dry. The plates are incubated at 30RC. The plates are incubated for 2 days and, at that time, numerous colonies could be seen growing on the agar. Some of these colonies are picked and subcultured onto nutrient agar plates. These apparent resistant cultures are tested for resistance by cross streaking with the phage lysate. A line of the phage lysate is streaked on the plate and allowed to dry. The presumptive resistant cultures are then streaked across the phage line. Resistant bacterial cultures show no lysis anywhere in the streak across the phage line after overnight incubation at 30RC. The resistance to phage is then reconfirmed by plating a lawn of the resistant culture onto a nutrient agar plate. The sensitive strain is also plated in the same manner to serve as the positive control. After drying, a drop of the phage lysate

is plated in the center of the plate and allowed to dry. Resistant cultures showed no lysis in the area where the phage lysate has been placed after incubation at 30RC for 24 hours.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 — Culturing *B.t.* Isolates and Transformed Hosts

A subculture of the *B.t.* isolates and transformed hosts of the invention can be used to inoculate the following medium, a peptone, glucose, salts medium.

Bacto Peptone	7.50 g/l
Glucose	1.00 g/l
KH_2PO_4	3.40 g/l
K_2HPO_4	4.35 g/l
Salt Solution	5.00 ml/l
$CaCl_2$ Solution	5.00 ml/l

Salts Solution (100 ml)

$MgSO_4 \cdot 7H_2O$	2.46 g
$MnSO_4 \cdot H_2O$	0.04 g
$ZnSO_4 \cdot 7H_2O$	0.28 g
$FeSO_4 \cdot 7H_2O$	0.40 g

$CaCl_2$ Solution (100 ml)

$CaCl_2 \cdot 2H_2O$	3.66 g

pH 7.2

The salts solution and $CaCl_2$ solution are filter-sterilized and added to the autoclaved and cooked broth at the time of inoculation. Flasks are incubated at 30RC on a rotary shaker at 200 rpm for 64 hours.

The above procedure can be readily scaled up to large fermentors by procedures well known in the art.

The *B.t.* spores and crystals, obtained in the above fermentation, can be isolated by procedures well known in the art. A frequently-used procedure is to subject the harvested fermentation broth to separation techniques, e.g., centrifugation.

Example 2 — Cloning of Novel Toxin Genes from *B.t.* Isolate PS50C

Total cellular DNA was prepared from *Bacillus thuringiensis* (*B.t.*) cells grown to an optical density, at 600 nm, of 1.0. The cells were recovered by centrifugation and protoplasts were prepared in TES buffer (30 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, pH = 8.0) containing 20% sucrose and 50 mg/ml lysozyme. The protoplasts were lysed by addition of SDS to a final concentration of 4%. The cellular material was precipitated overnight at 4RC in 100 mM (final concentration) neutral potassium chloride. The supernate was extracted twice with phenol/chloroform (1:1). Nucleic acids were precipitated with ethanol and DNA was purified by isopycnic banding on cesium chloride-ethidium bromide gradients.

Total cellular DNA from *B.t.* subsp. kumamotoensis (*B.t.kum.*), isolate PS50C, was digested with *Hin*dIII and fractionated by electrophoresis on a 0.8% (w/v) agarose-TAE (50 mM Tris-HCl, 20 mM NaOAc, 2.5 mM EDTA, pH = 8.0) buffered gel. A Southern blot of the gel was hybridized with a [^{32}P]-radiolabeled oligonucleotide probe. Results showed that the hybridizing fragments of PS50C are approximately 12 Kb and 1.7 Kb in size.

A library was constructed from PS50C total cellular DNA partially digested with *Sau*3A and size fractionated by gel electrophoresis. The 9-23 Kb region of the gel was excised and the DNA was electroeluted and then concentrated using an Elutip-d™ ion exchange column (Schleicher and Schuel, Keene, NH). The isolated *Sau*3A fragments were ligated into *Bam*HI-digested LambdaGEM-11™ (PROMEGA). The packaged phage were plated on *E. coli* KW251 cells (PROMEGA) at a high titer and screened using the radiolabeled oligonucleotide probe. Hybridizing plaques were purified and rescreened at a lower plaque density. Single isolated, purified plaques that hybridized with the probe were used to infect *E. coli* KW251 cells in liquid culture for preparation of phage for DNA isolation. DNA was isolated by standard procedures. Preparative amounts of DNA were digested with *Xho*I (to release the inserted DNA from lambda sequences) and separated by electrophoresis on a 0.6%

agarose-TAE gel. The large fragments were purified by ion exchange chromatography as above and ligated to *Xho*I-digested, dephosphorylated pHTBlueII (an *E. coli/B. thuringiensis* shuttle vector comprised of pBluescript s/k [Stratagene] and the replication origin from a resident *B.t.* plasmid [D. Lereclus *et al.* (1989) *FEMS Microbiology Letters* 60:211-218]). The ligation mix was introduced by transformation into competent *E. coli* NM522 cells (ATCC 47000) and plated on LB agar containing ampicillin, isopropyl-(β)-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-4-indolyl-(β)-D-galactoside (XGAL). White colonies, with putative restriction fragment insertions in the (β)-galactosidase gene of pHTBlueII, were subjected to standard rapid plasmid purification procedures. Plasmids were analyzed by *Xho*I digestion and agarose gel electrophoresis. The desired plasmid construct, pMYC1638, contains an approximately 12 Kb *Xho*I insert. A partial restriction map of the cloned insert indicates that the toxin gene is novel compared to the maps of other toxin genes encoding insecticidal proteins. The nucleotide sequence (open reading frame only), which has been designated 50C(a) is shown in SEQ ID NO. 1. The predicted peptide sequence of the toxin is shown in SEQ ID NO. 2.

Plasmid pMYC1638 was introduced into an acrystalliferous (Cry^-) *B.t.* host (HD-1 cryB obtained from A. Aronson, Purdue University) by electroporation. Expression of an approximately 130 kDa protein was verified by SDS-PAGE.

Plasmid pMYC1638 containing the *B.t.* toxin gene, can be removed from the transformed host microbe by use of standard well-known procedures. For example, *E. coli* NM522[pMYC1638] NRRL B-18751 can be subjected to cleared lysate isopycnic density gradient procedures, and the like, to recover pMYC1638.

A second gene, designated 50C(b), has also been cloned and sequenced from PS50C. The nucleotide sequence for 50C(a) is shown in SEQ ID NO. 3, and the predicted amino acid sequence for this toxin is shown in SEQ ID NO. 4.

Example 3 — Cloning of Toxin Gene From *B.t.* Isolate PS43F and Transformation into *Pseudomonas*

Total cellular DNA was prepared by growing the cells of *B.t.* isolate PS43F and M-7 to a low optical density (OD_{600} = 1.0) and recovering the cells by centrifugation. The cells were protoplasted in a buffer containing 20% sucrose and

50 mg/ml lysozyme. The protoplasts were lysed by addition of SDS to a final concentration of 4%. The cellular material was precipitated overnight at 4RC in 100 mM neutral potassium chloride. The supernate was phenol/chloroform extracted twice and the DNA precipitated in 68% ethanol. The DNA was purified on a cesium chloride gradient. DNAs from strains 43F and M-7 (as a standard of reference) were digested with *Eco*RI and run out on a 0.8% agarose gel. The gel was Southern blotted and probed with the nick translated ORF *Xmn*I to *Pst*I fragment of the toxin encoding gene isolated from M-7 (this will be subsequently referred to as Probe). The results showed 43F to hybridize to Probe at 7.5 kb which is different than the standard.

Preparative amounts of 43F DNA were digested with *Eco*RI and run out on a 0.8% agarose gel. The 7.5 kb region of the preparative gel was isolated and the DNA electroeluted and concentrated using an ELUTIP™-d (Schleicher and Schuell, Keene, NH) ion exchange column. A sample was blotted and probed to verify the fragment was indeed isolated. The 7.5 kb *Eco*RI fragment was ligated to Lambda ZAP™ *Eco*RI arms. The packaged recombinant phage were plated out with *E. coli* strain BB4 (Stratagene Cloning Systems, La Jolla, CA) to give high plaque density.

The plaques were screened by standard procedures with Probe. The plaques that hybridized were purified and re-screened at a lower plaque density. The resulting phage were grown with M13 helper phage (Stratagene) and the recombinant BLUESCRIPT™ plasmid was automatically excised and packaged. The "phagemid" was re-infected in XL1-blue *E. coli* cells (Stratagene) as part of the automatic excisi n process. The infected XL1-blue cells were screened for ampicillin resistance and the resulting colonies were miniprepped to find the desired plasmid pM1,98-4. The recombinant *E. coli* XL1-Blue (pM1,98-4) strain is called MR381.

The plasmid pM1,98-4 contained a 7.5 kb *Eco*RI insert. To verify that this insert was the one of interest, a Southern blot was performed and probed. The 7.5 kb band hybridized with Probe, confirming that the fragment had been cloned. Restriction endonuclease analysis of the 7.5 kb *Eco*RI fragment with the enzymes *Hin*dIII, *Pst*I, *Spe*I, *Bam*HI and *Xba*I was done to show that a gene different from M-7 had been cloned. The enzymes which cut inside the 7.5 kb *Eco*RI fragment were *Hin*dIII (twice) *Spe*I (twice) and *Pst*I (once). The open reading frame (ORF) of the 43F gene cut once with *Hin*dIII, twice with *Spe*I and did not cut with *Xba*I, *Eco*RI,

or *Bam*HI. The nucleotide sequence for the 43F gene is shown in SEQ ID NO. 5 and the predicted amino acid sequence for this toxin is provided in SEQ ID NO. 6.

The cloned toxin gene from PS43F can be modified for expression in *P. fluorescens* in the following way:

(1) A plasmid containing the P*tac*-promoted cryIA(b)-like toxin gene can be made using a 3-way ligation involving the P*tac* promoter and toxin gene on a *Bam*HI-*Pst*I fragment of about 4500 bp from pM3,130-7 (from MR420, NRRL B-18332, disclosed in U.S. Patent No. 5,055,294), a *Not*I-*Bam*HI fragment of about 5500 bp from pTJS260 (containing the tetracycline resistance genes, available from Dr. Donald Helinski, U.C. San Diego), and a *Not*I-*Pst*I fragment of about 6100 bp from pTJS260 (containing the replication region). The assembled plasmid is recovered following transformation of *E. coli* and growth under tetracycline selection.

(2) A plasmid containing the P*tac*-promoted 43F toxin gene can be made by ligating the toxin gene-containing *Fsp*I-*Ssp*I fragment of about 2200 bp from pM1,98-4 (from MR381(pM1,98-4), NRRL B-18291) into the *Sma*I site of the *E. coli* vector, pKK223-3 (Pharmacia). The P*tac*-promoted 43F toxin plasmid can be recovered following transformation of *E. coli*, growth under ampicillin selection, and screening for plasmids with inserts in the proper orientation for expression from the *tac* promoter by techniques well known in the art.

(3) The P*tac*-promoted 43F toxin can be assembled into, for example, the pTJS260-derived vector in a three-way ligation using the 12.6 kb DNA fragment having *Bam*HI and filled-in *Nsi*I ends from the plasmid resulting from step 1 above, to the *Bam*HI-*Nsi*I P*tac*-containing fragment of about 1.2 kb and the *Nsi*I-*Sca*I fragment of about 2.1 kb containing the 3Q end of the 43F toxin gene and adjacent vector DNA from the plasmid resulting from step 2 above.

The resulting pTJS260-derived 43F toxin expression plasmid can be introduced into *Pseudomonas fluorescens* by electroporation.

The above cloning procedures were conducted using standard procedures unless otherwise noted.

The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. These procedures are described in Maniatis, T., E.F. Fritsch, J. Sambrook (1982) *Molecular Cloning: A*

Laboratory Manual, Cold Spring Harbor Laboratory, New York. Thus, it is within the skill of those in the genetic engineering art to extract DNA from microbial cells, perform restriction enzyme digestions, electrophorese DNA fragments, tail and anneal plasmid and insert DNA, ligate DNA, transform cells, prepare plasmid DNA, electrophorese proteins, and sequence DNA.

The restriction enzymes disclosed herein can be purchased from Boehringer Mannheim, Indianapolis, IN, or New England BioLabs, Beverly, MA. The enzymes were used according to the instructions provided by the supplier.

Plasmid pM1,98-4 containing the *B.t.* toxin gene, can be removed from the transformed host microbe by use of standard well-known procedures. For example, *E. coli* XL1-Blue (pM1,98-4) can be subjected to cleared lysate isopycnic density gradient procedures, and the like, to recover pM1,98-4.

Example 4 — Testing of *B.t.* PS86B1 and Transformed Hosts

Third instar Pasadena Masked Chafers, *Cyclocephala pasadenae*, were found to be susceptible to the *B.t.* isolate PS86B1 as well as a *Pseudomonas fluorescens* transformed host containing the δ-endotoxin expressing gene obtained from *B.t.* PS43F. In the bioassays, larvae were fed an aqueous suspension of the material mixed with ryegrass roots. Larvae were held with the treated diet at room temperature in 1 oz. plastic cups, and observed for mortality by prodding. Dosages of PS86B1 and the *Pseudomonas fluorescens* transformed host greater than 500 ppm (δ-endotoxin protein/diet) gave 80% control in 15 days.

Example 5 - Testing of *B.t.* Transformed Host Containing a δ-Endotoxin Gene from PS50C

The transformed host was prepared by introducing plasmid pMYC1638 (NRRL B-18751), containing the δ-endotoxin expressing gene obtained from *B.t.* PS50C, into an acrystalliferous (cry$^-$) *B.t.* host (HD-1 cryB obtained from A. Aronson, Purdue University) by standard electroporation procedures.

Larvae of *Cotinis* sp. were found to be susceptible to the transformed host containing the δ-endotoxin expressing gene obtained from the *B.t.* isolate PS50C. The larvae were fed an aqueous suspension of the transformed host mixed with peat moss.

The larvae were held at room temperature in 1 oz. plastic cups with the treated peat, and checked regularly during the assays for mortality. Dosages of the transformed host of 750 ppm (δ-endotoxin/diet) caused 90% mortality of the larvae by day 13. In addition, the transformed host was shown to affect all three instar stages of the larvae.

Example 6 - Testing of *B.t.* PS86B1 Against *Cyclocephala borealis*

Third instar Northern Masked Chafer *Cyclocephala borealis* were found to be susceptible to the *B.t.* isolate PS86B1. Larvae were fed Kentucky bluegrass roots which had been dipped in a *B.t.* suspension. Larvae were held at room temperature in 1 oz. cups containing the treated roots and observed for mortality by prodding. Dosages greater than 500 ppm (protein/diet) gave 79% control in 7 days.

Example 7 - Testing of *B.t.* PS86B1 Against *Popillia japonica*

Third instar Japanese beetle *Popillia japonica* were found to be susceptible to the *B.t.* isolate PS86B1. Larvae were fed a *B.t.* suspension mixed with compost. Larvae were held with the treated compost at room temperature in 1 oz. plastic cups and observed for mortality by prodding. Dosages of PS86B1 greater than 500 ppm (protein/diet) gave greater than 40% control in 7 days.

Example 8 — Insertion of Toxin Genes Into Plants

One aspect of the subject invention is the transformation of plants with genes encoding a scarab-active toxin. The transformed plants are resistant to attack by scarab pests.

Genes encoding scarab-active toxins, as disclosed herein, can be inserted into plant cells using a variety of techniques which are well known in the art. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, etc. Accordingly, the sequence encoding the *B.t.* toxin can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid

is recovered. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be cleaved and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted.

The use of T-DNA for the transformation of plant cells has been intensively researched and sufficiently described in EP 120 516; Hoekema (1985) In: *The Binary Plant Vector System*, Offset-durkkerij Kanters B.V., Alblasserdam, Chapter 5; Fraley *et al.*, *Crit. Rev. Plant Sci.* 4:1-46; and An *et al.* (1985) *EMBO J.* 4:277-287.

Once the inserted DNA has been integrated in the genome, it is relatively stable there and, as a rule, does not come out again. It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G 418, bleomycin, hygromycin, or chloramphenicol, *inter alia*. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA.

A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, or electroporation as well as other possible methods. If agrobacteria are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the *vir* region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in agrobacteria. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in agrobacteria. They comprise a selection marker gene and a linker or polylinker which

are framed by the right and left T-DNA border regions. They can be transformed directly into agrobacteria (Holsters *et al.* [1978] *Mol. Gen. Genet.* 163:181-187). The agrobacterium used as host cell is to comprise a plasmid carrying a *vir* region. The *vir* region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can advantageously be cultivated with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

Example 9 — Cloning of Novel *B.t.* Genes Into Insect Viruses

A number of viruses are known to infect insects. These viruses include, for example, baculoviruses and entomopoxviruses. In one embodiment of the subject invention, lepidopteran-active genes, as described herein, can be placed with the genome of the insect virus, thus enhancing the pathogenicity of the virus. Methods for constructing insect viruses which comprise *B.t.* toxin genes are well known and readily practiced by those skilled in the art. These procedures are described, for example, in Merryweather *et al.* (Merryweather, A.T., U. Weyer, M.P.G. Harris, M. Hirst, T. Booth, R.D. Possee [1990] *J. Gen. Virol. 71*:1535-1544) and Martens *et al.* (Martens, J.W.M., G. Honee, D. Zuidema, J.W.M. van Lent, B. Visser, J.M. Vlak [1990] *Appl. Environmental Microbiol. 56*(9):2764-2770).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: MYCOGEN CORPORATION

(ii) TITLE OF INVENTION: Process for Controlling Scarab Pests with Bacillus thuringiensis Isolates

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: David R. Saliwanchik
(B) STREET: 2421 N.W. 41st Street, Suite A-1
(C) CITY: Gainesville
(D) STATE: FL
(E) COUNTRY: USA
(F) ZIP: 32606

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 07/828,430
(B) FILING DATE: 30-JAN-1992
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 07/808,316
(B) FILING DATE: 16-DEC-1991
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Saliwanchik, David R.
(B) REGISTRATION NUMBER: 31,794
(C) REFERENCE/DOCKET NUMBER: MA73.C2

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 904-375-8100
(B) TELEFAX: 904-372-5800

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3471 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus thuringiensis
(B) STRAIN: kumamotoensis

(C) INDIVIDUAL ISOLATE: PS50C

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: LAMBDAGEM (TM) - 11 LIBRARY OF LUIS FONCERRADA
(B) CLONE: 50C(a)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGAGTCCAA ATAATCAAAA TGAATATGAA ATTATAGATG CGACACCTTC TACATCTGTA 60

TCCAGTGATT CTAACAGATA CCCTTTTGCG AATGAGCCAA CAGATGCGTT ACAAAATATG 120

AATTATAAAG ATTATCTGAA AATGTCTGGG GGAGAGAATC CTGAATTATT TGGAAATCCG 180

GAGACGTTTA TTAGTTCATC CACGATTCAA ACTGGAATTG GCATTGTTGG TCGAATACTA 240

GGAGCTTTAG GGGTTCCATT TGCTAGTCAG ATAGCTAGTT TCTATAGTTT CATTGTTGGT 300

CAATTATGGC CGTCAAAGAG CGTAGATATA TGGGGAGAAA TTATGGAACG AGTGGAAGAA 360

CTCGTTGATC AAAAAATAGA AAAATATGTA AAAGATAAGG CTCTTGCTGA ATTAAAAGGG 420

CTAGGAAATG CTTTGGATGT ATATCAGCAG TCACTTGAAG ATTGGCTGGA AAATCGCAAT 480

GATGCAAGAA CTAGAAGTGT TGTTTCTAAT CAATTTATAG CTTTAGATCT TAACTTTGTT 540

AGTTCAATTC CATCTTTTGC AGTATCCGGA CACGAAGTAC TATTATTAGC AGTATATGCA 600

CAGGCTGTGA ACCTACATTT ATTGTTATTA AGAGATGCTT CTATTTTTGG AGAAGAGTGG 660

GGATTTACAC CAGGTGAAAT TTCTAGATTT TATAATCGTC AAGTGCAACT TACCGCTGAA 720

TATTCAGACT ATTGTGTAAA GTGGTATAAA ATCGGCTTAG ATAAATTGAA AGGTACCACT 780

TCTAAAAGTT GGCTGAATTA TCATCAGTTC CGTAGAGAGA TGACATTACT GGTATTAGAT 840

TTGGTGGCGT TATTTCCAAA CTATGACACA CATATGTATC CAATCGAAAC AACAGCTCAA 900

CTTACACGGG ATGTGTATAC AGATCCGATA GCATTTAACA TAGTGACAAG TACTGGATTC 960

TGCAACCCTT GGTCAACCCA CAGTGGTATT CTTTTTTATG AAGTTGAAAA CAACGTAATT 1020

CGTCCGCCAC ACTTGTTTGA TATACTCAGC TCAGTAGAAA TTAATACAAG TAGAGGGGGT 1080

ATTACGTTAA ATAATGATGC ATATATAAAC TACTGGTCAG GACATACCCT AAAATATCGT 1140

AGAACAGCTG ATTCGACCGT AACATACACA GCTAATTACG GTCGAATCAC TTCAGAAAAG 1200

AATTCATTTG CACTTGAGGA TAGGGATATT TTTGAAATTA ATTCAACTGT GGCAAACCTA 1260

GCTAATTACT ACCAAAAGGC ATATGGTGTG CCGGGATCTT GGTTCCATAT GGTAAAAAGG 1320

GGAACCTCAT CAACAACAGC GTATTTATAT TCAAAAACAC ATACAGCTCT CCAAGGGTGT 1380

ACACAGGTTT ATGAATCAAG TGATGAAATA CCTCTAGATA GAACTGTACC GGTAGCTGAA 1440

AGCTATAGTC ATAGATTATC TCATATTACC TCCCATTCTT TCTCTAAAAA TGGGAGTGCA 1500

TACTATGGGA GTTTCCCTGT ATTTGTTTGG ACACATACTA GTGCGGATTT AAATAATACA 1560

ATATATTCAG ATAAAATCAC TCAAATTCCA GCGGTAAAGG GAGACATGTT ATATCTAGGG 1620

GGTTCCGTAG TACAGGGTCC TGGATTTACA GGAGGAGATA TATTAAAAAG AACCAATCCT 1680

AGCATATTAG GGACCTTTGC GGTTACAGTA AATGGGTCGT TATCACAAAG ATATCGTGTA 1740

AGAATTCGCT ATGCCTCTAC AACAGATTTT GAATTTACTC TATACCTTGG CGACACAATA 1800

GAAAAAAATA GATTTAACAA AACTATGGAT AATGGGGCAT CTTTAACGTA TGAAACATTT 1860

AAATTCGCAA GTTTCATTAC TGATTTCCAA TTCAGAGAAA CACAAGATAA AATACTCCTA 1920

TCCATGGGTG ATTTTAGCTC CGGTCAAGAA GTTTATATAG ACCGAATCGA ATTCATCCCA 1980

GTAGATGAGA CATATGAGGC GGAACAAGAT TTAGAAGCGG CGAAGAAAGC AGTGAATGCC 2040

TTGTTTACGA ATACAAAAGA TGGCTTACGA CCAGGTGTAA CGGATTATGA AGTAAATCAA 2100

GCGGCAAACT TAGTGGAATG CCTATCGGAT GATTTATATC CAAATGAAAA ACGATTGTTA 2160

TTTGATGCGG TGAGAGAGGC AAAACGCCTC AGTGGGGCAC GTAACTTACT ACAAGATCCA 2220

GATTTCCAAG AGATAAACGG AGAAAATGGA TGGGCGGCAA GTACGGGAAT TGAGATTGTA 2280

GAAGGGGATG CTGTATTTAA AGGACGTTAT CTACGCCTAC CAGGTGCACG AGAAATTGAT 2340

ACGGAAACGT ATCCAACGTA TCTGTATCAA AAAGTAGAGG AAGGTGTATT AAAACCATAC 2400

ACAAGATATA GACTGAGAGG GTTTGTGGGA AGTAGTCAAG GATTAGAAAT TTATACGATA 2460

CGTCACCAAA CGAATCGAAT TGTAAAGAAT GTACCAGATG ATTTATTGCC AGATGTATCT 2520

CCTGTAAACT CTGATGGCAG TATCAATCGA TGCAGCGAAC AAAAGTATGT GAATAGCCGT 2580

TTAGAAGGAG AAAACCGTTC TGGTGATGCA CATGAGTTCT CGCTCCCTAT CGATATAGGA 2640

GAGCTGGATT ACAATGAAAA TGCAGGAATA TGGGTTGGAT TTAAGATTAC GGACCCAGAG 2700

GGATACGCAA CACTTGGAAA TCTTGAATTA GTCGAAGAGG GACCTTTGTC AGGAGACGCA 2760

TTAGAGCGCT TGCAAAGAGA AGAACAACAG TGGAAGATTC AAATGACAAG AAGACGTGAA 2820

GAGACAGATA GAAGATACAT GGCATCGAAA CAAGCGGTAG ATCGTTTATA TGCCGATTAT 2880

CAGGATCAAC AACTGAATCC TGATGTAGAG ATTACAGATC TTACTGCGGC TCAAGATCTG 2940

ATACAGTCCA TTCCTTACGT ATATAACGAA ATGTTCCCAG AAATACCAGG GATGAACTAT 3000

ACGAAGTTTA CAGAATTAAC AGATCGACTC CAACAAGCGT GGAATTTGTA TGATCAGCGA 3060

AATGCCATAC CAAATGGTGA TTTTCGAAAT GGGTTAAGTA ATTGGAATGC AACGCCTGGC 3120

GTAGAAGTAC AACAAATCAA TCATACATCT GTCCTTGTGA TTCCAAACTG GGATGAACAA 3180

GTTTCACAAC AGTTTACAGT TCAACCGAAT CAAAGATATG TATTACGAGT TACTGCAAGA 3240

AAAGAAGGGG TAGGAAATGG ATATGTAAGT ATTCGTGATG GTGGAAATCA ATCAGAAACG 3300

CTTACTTTTA GTGCAAGCGA TTATGATACA AATGGTGTGT ATAATGACCA AACCGGCTAT 3360

ATCACAAAAA CAGTGACATT CATCCCGTAT ACAGATCAAA TGTGGATTGA AATAAGTGAA 3420

ACAGAAGGTA CGTTCTATAT AGAAAGTGTA GAATTGATTG TAGACGTAGA G 3471

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1157 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus thuringiensis
(B) STRAIN: kumamotoensis
(C) INDIVIDUAL ISOLATE: PS50C

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Lambdagem (TM) - 11 LIBRARY OF LUIS FONCERRADA
(B) CLONE: 50C(a)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ser Pro Asn Asn Gln Asn Glu Tyr Glu Ile Ile Asp Ala Thr Pro
1 5 10 15

Ser Thr Ser Val Ser Ser Asp Ser Asn Arg Tyr Pro Phe Ala Asn Glu
20 25 30

Pro Thr Asp Ala Leu Gln Asn Met Asn Tyr Lys Asp Tyr Leu Lys Met
35 40 45

Ser Gly Gly Glu Asn Pro Glu Leu Phe Gly Asn Pro Glu Thr Phe Ile
50 55 60

Ser Ser Ser Thr Ile Gln Thr Gly Ile Gly Ile Val Gly Arg Ile Leu
65 70 75 80

Gly Ala Leu Gly Val Pro Phe Ala Ser Gln Ile Ala Ser Phe Tyr Ser
85 90 95

Phe Ile Val Gly Gln Leu Trp Pro Ser Lys Ser Val Asp Ile Trp Gly
100 105 110

Glu Ile Met Glu Arg Val Glu Glu Leu Val Asp Gln Lys Ile Glu Lys
115 120 125

Tyr Val Lys Asp Lys Ala Leu Ala Glu Leu Lys Gly Leu Gly Asn Ala
130 135 140

Leu Asp Val Tyr Gln Gln Ser Leu Glu Asp Trp Leu Glu Asn Arg Asn
145 150 155 160

Asp Ala Arg Thr Arg Ser Val Val Ser Asn Gln Phe Ile Ala Leu Asp
165 170 175

Leu Asn Phe Val Ser Ser Ile Pro Ser Phe Ala Val Ser Gly His Glu
180 185 190

Val Leu Leu Leu Ala Val Tyr Ala Gln Ala Val Asn Leu His Leu Leu
195 200 205

Leu Leu Arg Asp Ala Ser Ile Phe Gly Glu Glu Trp Gly Phe Thr Pro
210 215 220

Gly Glu Ile Ser Arg Phe Tyr Asn Arg Gln Val Gln Leu Thr Ala Glu
225 230 235 240

Tyr Ser Asp Tyr Cys Val Lys Trp Tyr Lys Ile Gly Leu Asp Lys Leu
245 250 255

Lys Gly Thr Thr Ser Lys Ser Trp Leu Asn Tyr His Gln Phe Arg Arg
260 265 270

Glu Met Thr Leu Leu Val Leu Asp Leu Val Ala Leu Phe Pro Asn Tyr
275 280 285

Asp Thr His Met Tyr Pro Ile Glu Thr Thr Ala Gln Leu Thr Arg Asp
290 295 300

Val Tyr Thr Asp Pro Ile Ala Phe Asn Ile Val Thr Ser Thr Gly Phe
305 310 315 320

Cys Asn Pro Trp Ser Thr His Ser Gly Ile Leu Phe Tyr Glu Val Glu
325 330 335

Asn Asn Val Ile Arg Pro Pro His Leu Phe Asp Ile Leu Ser Ser Val
340 345 350

Glu Ile Asn Thr Ser Arg Gly Gly Ile Thr Leu Asn Asn Asp Ala Tyr
355 360 365

Ile Asn Tyr Trp Ser Gly His Thr Leu Lys Tyr Arg Arg Thr Ala Asp
370 375 380

Ser Thr Val Thr Tyr Thr Ala Asn Tyr Gly Arg Ile Thr Ser Glu Lys
385 390 395 400

Asn Ser Phe Ala Leu Glu Asp Arg Asp Ile Phe Glu Ile Asn Ser Thr
405 410 415

Val Ala Asn Leu Ala Asn Tyr Tyr Gln Lys Ala Tyr Gly Val Pro Gly
420 425 430

Ser Trp Phe His Met Val Lys Arg Gly Thr Ser Ser Thr Thr Ala Tyr
435 440 445

Leu Tyr Ser Lys Thr His Thr Ala Leu Gln Gly Cys Thr Gln Val Tyr
450 455 460

Glu Ser Ser Asp Glu Ile Pro Leu Asp Arg Thr Val Pro Val Ala Glu
465 470 475 480

Ser Tyr Ser His Arg Leu Ser His Ile Thr Ser His Ser Phe Ser Lys
485 490 495

Asn Gly Ser Ala Tyr Tyr Gly Ser Phe Pro Val Phe Val Trp Thr His
500 505 510

Thr Ser Ala Asp Leu Asn Asn Thr Ile Tyr Ser Asp Lys Ile Thr Gln
515 520 525

Ile Pro Ala Val Lys Gly Asp Met Leu Tyr Leu Gly Gly Ser Val Val
530 535 540

Gln Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu Lys Arg Thr Asn Pro
545 550 555 560

Ser Ile Leu Gly Thr Phe Ala Val Thr Val Asn Gly Ser Leu Ser Gln
565 570 575

Arg Tyr Arg Val Arg Ile Arg Tyr Ala Ser Thr Thr Asp Phe Glu Phe
580 585 590

Thr Leu Tyr Leu Gly Asp Thr Ile Glu Lys Asn Arg Phe Asn Lys Thr
595 600 605

Met Asp Asn Gly Ala Ser Leu Thr Tyr Glu Thr Phe Lys Phe Ala Ser
610 615 620

Phe Ile Thr Asp Phe Gln Phe Arg Glu Thr Gln Asp Lys Ile Leu Leu
625 630 635 640

Ser Met Gly Asp Phe Ser Ser Gly Gln Glu Val Tyr Ile Asp Arg Ile
645 650 655

Glu Phe Ile Pro Val Asp Glu Thr Tyr Glu Ala Glu Gln Asp Leu Glu
660 665 670

Ala Ala Lys Lys Ala Val Asn Ala Leu Phe Thr Asn Thr Lys Asp Gly
675 680 685

Leu Arg Pro Gly Val Thr Asp Tyr Glu Val Asn Gln Ala Ala Asn Leu
690 695 700

Val Glu Cys Leu Ser Asp Asp Leu Tyr Pro Asn Glu Lys Arg Leu Leu
705 710 715 720

Phe Asp Ala Val Arg Glu Ala Lys Arg Leu Ser Gly Ala Arg Asn Leu
725 730 735

Leu Gln Asp Pro Asp Phe Gln Glu Ile Asn Gly Glu Asn Gly Trp Ala
740 745 750

Ala Ser Thr Gly Ile Glu Ile Val Glu Gly Asp Ala Val Phe Lys Gly
755 760 765

Arg Tyr Leu Arg Leu Pro Gly Ala Arg Glu Ile Asp Thr Glu Thr Tyr
770 775 780

Pro Thr Tyr Leu Tyr Gln Lys Val Glu Glu Gly Val Leu Lys Pro Tyr
785 790 795 800

Thr Arg Tyr Arg Leu Arg Gly Phe Val Gly Ser Ser Gln Gly Leu Glu
805 810 815

Ile Tyr Thr Ile Arg His Gln Thr Asn Arg Ile Val Lys Asn Val Pro
820 825 830

Asp Asp Leu Leu Pro Asp Val Ser Pro Val Asn Ser Asp Gly Ser Ile
835 840 845

Asn Arg Cys Ser Glu Gln Lys Tyr Val Asn Ser Arg Leu Glu Gly Glu
850 855 860

Asn Arg Ser Gly Asp Ala His Glu Phe Ser Leu Pro Ile Asp Ile Gly
865 870 875 880

Glu Leu Asp Tyr Asn Glu Asn Ala Gly Ile Trp Val Gly Phe Lys Ile
885 890 895

Thr Asp Pro Glu Gly Tyr Ala Thr Leu Gly Asn Leu Glu Leu Val Glu
900 905 910

Glu Gly Pro Leu Ser Gly Asp Ala Leu Glu Arg Leu Gln Arg Glu Glu
915 920 925

Gln Gln Trp Lys Ile Gln Met Thr Arg Arg Arg Glu Glu Thr Asp Arg
930 935 940

Arg Tyr Met Ala Ser Lys Gln Ala Val Asp Arg Leu Tyr Ala Asp Tyr
945 950 955 960

Gln Asp Gln Gln Leu Asn Pro Asp Val Glu Ile Thr Asp Leu Thr Ala
965 970 975

Ala Gln Asp Leu Ile Gln Ser Ile Pro Tyr Val Tyr Asn Glu Met Phe
980 985 990

Pro Glu Ile Pro Gly Met Asn Tyr Thr Lys Phe Thr Glu Leu Thr Asp
995 1000 1005

Arg Leu Gln Gln Ala Trp Asn Leu Tyr Asp Gln Arg Asn Ala Ile Pro
1010 1015 1020

Asn Gly Asp Phe Arg Asn Gly Leu Ser Asn Trp Asn Ala Thr Pro Gly
1025 1030 1035 1040

Val Glu Val Gln Gln Ile Asn His Thr Ser Val Leu Val Ile Pro Asn
1045 1050 1055

Trp Asp Glu Gln Val Ser Gln Gln Phe Thr Val Gln Pro Asn Gln Arg
1060 1065 1070

Tyr Val Leu Arg Val Thr Ala Arg Lys Glu Gly Val Gly Asn Gly Tyr
1075 1080 1085

Val Ser Ile Arg Asp Gly Gly Asn Gln Ser Glu Thr Leu Thr Phe Ser
1090 1095 1100

Ala Ser Asp Tyr Asp Thr Asn Gly Val Tyr Asn Asp Gln Thr Gly Tyr
1105 1110 1115 1120

Ile Thr Lys Thr Val Thr Phe Ile Pro Tyr Thr Asp Gln Met Trp Ile
1125 1130 1135

Glu Ile Ser Glu Thr Glu Gly Thr Phe Tyr Ile Glu Ser Val Glu Leu
1140 1145 1150

Ile Val Asp Val Glu
1155

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3507 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus thuringiensis
(B) STRAIN: kumamotoensis
(C) INDIVIDUAL ISOLATE: 50C

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: LambdaGEM-11(tm) library of L. Foncerrada
(B) CLONE: 50C(b)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGAGTCCAA ATAATCAAAA TGAATATGAA ATTATAGATG CGACACCTTC TACATCTGTA 60

TCCAATGATT CTAACAGATA CCCTTTTGCG AATGAGCCAA CAAATGCGCT ACAAAATATG 120

GATTATAAAG ATTATTTAAA AATGTCTGCG GGAAATGTTA GTGAATACCC TGGTTCACCT 180

GAGGTATTTC TAAGCGAGCA AGATGCAGTT AAGGCCGCAA TTGATATAGT AGGTAAATTA 240

CTAACAGGTT TAGGGGTTCC ATTTGTTGGG CCGATAGTGA GTCTTTATAC TCAACTTATT 300

GATATTCTGT GGCCTTCAAA ACAAAAGAGT CAATGGGAAA TTTTTATGGA ACAAGTAGAA 360

GAACTCATTA ATCAAAAAAT AGCAGAATAT GCAAGGAATA AAGCGCTTTC GGAATTGGAA 420

GGGCTAGGGA ATAATTACCA ATTATATCTA ACTGCGCTTG AAGAGTGGAA AGAAAATCCA 480

AATGGTTCAA GAGCCTTACG AGATGTTCGA AATCGATTTG AAATCCTGGA TAGTTTATTT 540

ACGCAATATA TGCCATCTTT TCGAGTGACA AATTTTGAAG TACCATTCCT TACAGTATAT 600

ACAATGGCAG CAAACCTACA TTTACTTTTA TTAAGGGACG CATCAATTTT TGGAGAAGAA 660

TGGGGATTGT CTACAAGCAC TATTAATAAC TACTATAATC GTCAAATGAA ACTTACTGCA 720

GAATATTCTG ACCACTGTGT AAAGTGGTAT GAAACTGGTT TAGCAAAATT AAAAGGCTCG 780

AGCGCTAAAC AATGGATTGA CTATAACCAA TTCCGTAGAG AAATGACATT GACGGTGTTA 840

GACGTTGTTG CATTATTTTC AAACTATGAT ACGCGTACGT ATCCACTGGC AACAACAGCT 900

CAGCTTACAA GGGAAGTATA TACAGATCCA CTTGGCGCGG TAGATGTGCC TAATATTGGC 960

TCCTGGTATG ACAAAGCACC TTCTTTCTCA GAAATAGAAA AAGCGGCTAT TCGTCCACCT 1020

CATGTGTTTG ATTATATAAC GGGACTCACA GTTTATACAA AAAAACGTAG CTTCACTTCT 1080

GATCGTTATA TGAGATATTG GGCTGGTCAT CAAATAAGCT ATAAGCATAT CGGTACGAGT 1140

AGTACCTTTA CACAGATGTA TGGAACCAAT CAAAATTTAC AAAGTACTAG CAATTTTGAT 1200

TTTACGAATT ACGATATTTA CAAGACTTTA TCAAATGGTG CAGTACTCCT TGATATAGTT 1260

TACCCTGGTT ATACGTATAC ATTTTTTGGA ATGCCAGAAA CCGAGTTTTT TATGGTAAAT 1320

CAATTGAATA ATACCAGAAA GACGTTAACG TATAAACCAG CTTCCAAAGA TATTATAGAT 1380

CGGACAAGAG ATTCGGAATT AGAATTGCCT CCAGAAACTT CAGGTCAACC AAATTACGAG 1440

TCATATAGCC ATAGATTAGG TCATATTACA TTTATTTACT CCAGTTCAAC TAGCACGTAT 1500

GTACCTGTAT TTTCTTGGAC ACATCGGAGT GCAGATCTAA CAAATACAGT TAAAAGTGGC 1560

GAAATCACCC AAATACCAGG GGGCAAGTCT AGCACCATAG GCAGAAATAC TTATATAATA 1620

AAAGGGCGTG GTTATACAGG GGGAGACTTA GTGGCTTTAA CGGACCGCAT CGGAAGTTGT 1680

GAGTTTCAGA TGATCTTTCC AGAGTCTCAA CGATTCCGTA TTCGGATTCG TTACGCTTCT 1740

AATGAAACTA GTTATATTAG TTTATACGGA CTAAACCAAA GCGGAACTTT AAAATTCAAC 1800

CAGACATATT CTAATAAAAA TGAAAATGAT TTAACATATA ATGATTTCAA ATATATAGAA 1860

TATCCAAGAG TCATTTCAGT AAATGCTTCT TCAAACATAC AGAGGTTATC TATAGGTATA 1920

CAAACGAATA CAAATTTATT TATTTTAGAC CGAATCGAAT TCATCCCAGT AGATGAGACA 1980

TATGAAGCGG AAACGGATTT AGAAGCGGCA AAGAAAGCAG TGAATGCCTT GTTTACGAAT 2040

ACAAAAGATG GATTACAGCC AGGTGTAACG GATTATGAAG TAAATCAAGC GGCCAACTTA 2100

GTGGAATGCC TATCGGATGA TTTGTATCCA AATGAAAAAC GATTGTTATT TGATGCAGTG 2160

AGAGAGGCAA AACGACTTAG CGAGGCACGG AACTTACTAC AAGATCCAGA TTTCCAAGAG 2220

ATAAATGGAG AAAATGGATG GACGGCAAGT ACGGGAATTG AGGTTATAGA AGGGGATGCT 2280

GTATTCAAAG GGCGTTATCT ACGCCTACCA GGTGCGAGAG AAATAGATAC GGAAACGTAT 2340

CCAACGTATC TGTATCAAAA AGTAGAGGAA GGTGTATTAA AACCATACAC AAGGTATAGA 2400

CTGAGAGGAT TTGTGGGAAG TAGTCAAGGA TTAGAAATTT ATACGATTCG TCACCAAACG 2460

AATCGAATTG TAAAAAATGT ACCAGATGAT TTACTGCCAG ATGTACCTCC TGTAAACAAT 2520

GATGGTAGAA TCAATCGATG CAGCGAACAA AAGTATGTGA ATAGTCGTTT AGAAGTAGAA 2580

AACCGTTCTG GTGAAGCGCA TGAGTTCTCA ATCCCTATCG ATACAGGAGA GCTGGATTAC 2640

AATGAAAATG CAGGAATATG GGTTGGATTT AAGATTACGG ACCCAGAGGG ATACGCAACA 2700

CTTGGAAATC TTGAATTGGT CGAAGAGGGA CCTTTGTCAG GAGACGCATT AGAACGCTTG 2760

CAAAAAGAAG AACAACAGTG GAAGATTCAA ATGACAAGAA GACGTGAAGA GACAGATAGA 2820

AGATACATGG CATCGAAACA AGCGGTAGAT CGTTTATATG CCGATTATCA GGATCAGCAA 2880

CTGAATCCGA ATGTAGAGAT TACAGATCTT ACTGCGGCTC AAGATCTAAT ACAGTCCATT 2940

CCTTACGTGT ATAACGAAAT GTTCCCAGAA ATACCAGGAA TGAACTATAC GAAGTTTACA 3000

GAGTTAACAG ATCGACTCCA ACAAGCCTGG GGATTGTATG ATCAACGAAA CGCTATACCA 3060

AATGGAGATT ACCGAAATGA ATTAAGTAAT TGGAATACAA CATCTGGTGT GAATGTACAA 3120

CAAATCAATC ATACATCTGT CCTTGTGATT CCAAACTGGA ATGAACAAGT TTCACAAAAG 3180

TTTACAGTTC AACCGAATCA AAGATATGTG TTACGAGTTA CTGCAAGAAA AGAAGGGGTA 3240

GGAAATGGAT ATGTAAGTAT TCGTGATGGT GGAAATCAAT CAGAAACGCT TACTTTTAGT 3300

GCAAGCGATT ATGATACAAA TGGTATGTAT GATACACAAG CGTCGAATAC AAACGGATAT 3360

AACACAAATA GTGTGTACAT GATCAAACCG GCTATATCAC GAAAAACAGT GGACATTTCA 3420

TCCGTATACA ATCAAATGTG GATTGAGATA AGTGAGACAG AAGGTACGTT CTATATAGAA 3480

AGTGTAGAAT TGATTGTAGA CGTAGAG 3507

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1169 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus thuringiensis
(B) STRAIN: kumamotoensis
(C) INDIVIDUAL ISOLATE: 50C

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: LambdaGEM-11 library of L. Foncerrada
(B) CLONE: 50C(b)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ser Pro Asn Asn Gln Asn Glu Tyr Glu Ile Ile Asp Ala Thr Pro
1 5 10 15

Ser Thr Ser Val Ser Asn Asp Ser Asn Arg Tyr Pro Phe Ala Asn Glu
20 25 30

```
Pro Thr Asn Ala Leu Gln Asn Met Asp Tyr Lys Asp Tyr Leu Lys Met
        35                  40                  45

Ser Ala Gly Asn Val Ser Glu Tyr Pro Gly Ser Pro Glu Val Phe Leu
    50                  55                  60

Ser Glu Gln Asp Ala Val Lys Ala Ala Ile Asp Ile Val Gly Lys Leu
65                  70                  75                  80

Leu Thr Gly Leu Gly Val Pro Phe Val Gly Pro Ile Val Ser Leu Tyr
                85                  90                  95

Thr Gln Leu Ile Asp Ile Leu Trp Pro Ser Lys Gln Lys Ser Gln Trp
            100                 105                 110

Glu Ile Phe Met Glu Gln Val Glu Glu Leu Ile Asn Gln Lys Ile Ala
        115                 120                 125

Glu Tyr Ala Arg Asn Lys Ala Leu Ser Glu Leu Glu Gly Leu Gly Asn
    130                 135                 140

Asn Tyr Gln Leu Tyr Leu Thr Ala Leu Glu Glu Trp Lys Glu Asn Pro
145                 150                 155                 160

Asn Gly Ser Arg Ala Leu Arg Asp Val Arg Asn Arg Phe Glu Ile Leu
                165                 170                 175

Asp Ser Leu Phe Thr Gln Tyr Met Pro Ser Phe Arg Val Thr Asn Phe
            180                 185                 190

Glu Val Pro Phe Leu Thr Val Tyr Thr Met Ala Ala Asn Leu His Leu
        195                 200                 205

Leu Leu Leu Arg Asp Ala Ser Ile Phe Gly Glu Glu Trp Gly Leu Ser
    210                 215                 220

Thr Ser Thr Ile Asn Asn Tyr Tyr Asn Arg Gln Met Lys Leu Thr Ala
225                 230                 235                 240

Glu Tyr Ser Asp His Cys Val Lys Trp Tyr Glu Thr Gly Leu Ala Lys
                245                 250                 255

Leu Lys Gly Ser Ser Ala Lys Gln Trp Ile Asp Tyr Asn Gln Phe Arg
            260                 265                 270

Arg Glu Met Thr Leu Thr Val Leu Asp Val Val Ala Leu Phe Ser Asn
        275                 280                 285

Tyr Asp Thr Arg Thr Tyr Pro Leu Ala Thr Thr Ala Gln Leu Thr Arg
    290                 295                 300

Glu Val Tyr Thr Asp Pro Leu Gly Ala Val Asp Val Pro Asn Ile Gly
305                 310                 315                 320

Ser Trp Tyr Asp Lys Ala Pro Ser Phe Ser Glu Ile Glu Lys Ala Ala
                325                 330                 335

Ile Arg Pro Pro His Val Phe Asp Tyr Ile Thr Gly Leu Thr Val Tyr
            340                 345                 350

Thr Lys Lys Arg Ser Phe Thr Ser Asp Arg Tyr Met Arg Tyr Trp Ala
        355                 360                 365

Gly His Gln Ile Ser Tyr Lys His Ile Gly Thr Ser Ser Thr Phe Thr
    370                 375                 380

Gln Met Tyr Gly Thr Asn Gln Asn Leu Gln S r Thr Ser Asn Phe Asp
385                 390                 395                 400
```

Phe Thr Asn Tyr Asp Ile Tyr Lys Thr Leu Ser Asn Gly Ala Val Leu
405 410 415

Leu Asp Ile Val Tyr Pro Gly Tyr Thr Tyr Thr Phe Phe Gly Met Pro
420 425 430

Glu Thr Glu Phe Phe Met Val Asn Gln Leu Asn Asn Thr Arg Lys Thr
435 440 445

Leu Thr Tyr Lys Pro Ala Ser Lys Asp Ile Ile Asp Arg Thr Arg Asp
450 455 460

Ser Glu Leu Glu Leu Pro Pro Glu Thr Ser Gly Gln Pro Asn Tyr Glu
465 470 475 480

Ser Tyr Ser His Arg Leu Gly His Ile Thr Phe Ile Tyr Ser Ser Ser
485 490 495

Thr Ser Thr Tyr Val Pro Val Phe Ser Trp Thr His Arg Ser Ala Asp
500 505 510

Leu Thr Asn Thr Val Lys Ser Gly Glu Ile Thr Gln Ile Pro Gly Gly
515 520 525

Lys Ser Ser Thr Ile Gly Arg Asn Thr Tyr Ile Ile Lys Gly Arg Gly
530 535 540

Tyr Thr Gly Gly Asp Leu Val Ala Leu Thr Asp Arg Ile Gly Ser Cys
545 550 555 560

Glu Phe Gln Met Ile Phe Pro Glu Ser Gln Arg Phe Arg Ile Arg Ile
565 570 575

Arg Tyr Ala Ser Asn Glu Thr Ser Tyr Ile Ser Leu Tyr Gly Leu Asn
580 585 590

Gln Ser Gly Thr Leu Lys Phe Asn Gln Thr Tyr Ser Asn Lys Asn Glu
595 600 605

Asn Asp Leu Thr Tyr Asn Asp Phe Lys Tyr Ile Glu Tyr Pro Arg Val
610 615 620

Ile Ser Val Asn Ala Ser Ser Asn Ile Gln Arg Leu Ser Ile Gly Ile
625 630 635 640

Gln Thr Asn Thr Asn Leu Phe Ile Leu Asp Arg Ile Glu Phe Ile Pro
645 650 655

Val Asp Glu Thr Tyr Glu Ala Glu Thr Asp Leu Glu Ala Ala Lys Lys
660 665 670

Ala Val Asn Ala Leu Phe Thr Asn Thr Lys Asp Gly Leu Gln Pro Gly
675 680 685

Val Thr Asp Tyr Glu Val Asn Gln Ala Ala Asn Leu Val Glu Cys Leu
690 695 700

Ser Asp Asp Leu Tyr Pro Asn Glu Lys Arg Leu Leu Phe Asp Ala Val
705 710 715 720

Arg Glu Ala Lys Arg Leu Ser Glu Ala Arg Asn Leu Leu Gln Asp Pro
725 730 735

Asp Phe Gln Glu Ile Asn Gly Glu Asn Gly Trp Thr Ala Ser Thr Gly
740 745 750

Ile Glu Val Ile Glu Gly Asp Ala Val Phe Lys Gly Arg Tyr Leu Arg
755 760 765

Leu Pro Gly Ala Arg Glu Ile Asp Thr Glu Thr Tyr Pro Thr Tyr Leu
770 775 780

Tyr Gln Lys Val Glu Glu Gly Val Leu Lys Pro Tyr Thr Arg Tyr Arg
785 790 795 800

Leu Arg Gly Phe Val Gly Ser Ser Gln Gly Leu Glu Ile Tyr Thr Ile
805 810 815

Arg His Gln Thr Asn Arg Ile Val Lys Asn Val Pro Asp Asp Leu Leu
820 825 830

Pro Asp Val Pro Pro Val Asn Asn Asp Gly Arg Ile Asn Arg Cys Ser
835 840 845

Glu Gln Lys Tyr Val Asn Ser Arg Leu Glu Val Glu Asn Arg Ser Gly
850 855 860

Glu Ala His Glu Phe Ser Ile Pro Ile Asp Thr Gly Glu Leu Asp Tyr
865 870 875 880

Asn Glu Asn Ala Gly Ile Trp Val Gly Phe Lys Ile Thr Asp Pro Glu
885 890 895

Gly Tyr Ala Thr Leu Gly Asn Leu Glu Leu Val Glu Glu Gly Pro Leu
900 905 910

Ser Gly Asp Ala Leu Glu Arg Leu Gln Lys Glu Glu Gln Gln Trp Lys
915 920 925

Ile Gln Met Thr Arg Arg Arg Glu Glu Thr Asp Arg Arg Tyr Met Ala
930 935 940

Ser Lys Gln Ala Val Asp Arg Leu Tyr Ala Asp Tyr Gln Asp Gln Gln
945 950 955 960

Leu Asn Pro Asn Val Glu Ile Thr Asp Leu Thr Ala Ala Gln Asp Leu
965 970 975

Ile Gln Ser Ile Pro Tyr Val Tyr Asn Glu Met Phe Pro Glu Ile Pro
980 985 990

Gly Met Asn Tyr Thr Lys Phe Thr Glu Leu Thr Asp Arg Leu Gln Gln
995 1000 1005

Ala Trp Gly Leu Tyr Asp Gln Arg Asn Ala Ile Pro Asn Gly Asp Tyr
1010 1015 1020

Arg Asn Glu Leu Ser Asn Trp Asn Thr Thr Ser Gly Val Asn Val Gln
1025 1030 1035 1040

Gln Ile Asn His Thr Ser Val Leu Val Ile Pro Asn Trp Asn Glu Gln
1045 1050 1055

Val Ser Gln Lys Phe Thr Val Gln Pro Asn Gln Arg Tyr Val Leu Arg
1060 1065 1070

Val Thr Ala Arg Lys Glu Gly Val Gly Asn Gly Tyr Val Ser Ile Arg
1075 1080 1085

Asp Gly Gly Asn Gln Ser Glu Thr Leu Thr Phe Ser Ala Ser Asp Tyr
1090 1095 1100

Asp Thr Asn Gly Met Tyr Asp Thr Gln Ala Ser Asn Thr Asn Gly Tyr
1105 1110 1115 1120

Asn Thr Asn Ser Val Tyr M t Ile Lys Pro Ala Ile Ser Arg Lys Thr
1125 1130 1135

Val Asp Ile Ser Ser Val Tyr Asn Gln Met Trp Ile Glu Ile Ser Glu
1140 1145 1150

Thr Glu Gly Thr Phe Tyr Ile Glu Ser Val Glu Leu Ile Val Asp Val
1155 1160 1165

Glu

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1953 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus thuringiensis
(B) STRAIN: tolworthi
(C) INDIVIDUAL ISOLATE: 43F

(vii) IMMEDIATE SOURCE:
(B) CLONE: E. coli XLI-Blue (pM1,98-4), NRRL B-18291

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1953

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
ATG AAT CCA AAC AAT CGA AGT GAA TAT GAT ACG ATA AAG GTT ACA CCT        48
M t Asn Pro Asn Asn Arg Ser Glu Tyr Asp Thr Ile Lys Val Thr Pro
  1               5                  10                  15

AAC AGT GAA TTG CCA ACT AAC CAT AAT CAA TAT CCT TTA GCT GAC AAT        96
Asn Ser Glu Leu Pro Thr Asn His Asn Gln Tyr Pro Leu Ala Asp Asn
            20                  25                  30

CCA AAT TCG ACA CTA GAA GAA TTA AAT TAT AAA GAA TTT TTA AGA ATG       144
Pro Asn Ser Thr Leu Glu Glu Leu Asn Tyr Lys Glu Phe Leu Arg Met
        35                  40                  45

ACT GCA GAC AAT TCT ACG GAA GTG CTA GAC AGC TCT ACA GTA AAA GAT       192
Thr Ala Asp Asn Ser Thr Glu Val Leu Asp Ser Ser Thr Val Lys Asp
    50                  55                  60

GCA GTT GGG ACA GGA ATT TCT GTT GTA GGA CAG ATT TTA GGT GTT GTA       240
Ala Val Gly Thr Gly Ile Ser Val Val Gly Gln Ile Leu Gly Val Val
65                  70                  75                  80

GGG GTT CCA TTT GCT GGG GCG CTC ACT TCA TTT TAT CAA TCA TTT CTT       288
Gly Val Pro Phe Ala Gly Ala Leu Thr Ser Phe Tyr Gln Ser Phe Leu
                85                  90                  95

AAC GCT ATA TGG CCA AGT GAT GCT GAC CCA TGG AAG GCT TTT ATG GCA       336
Asn Ala Ile Trp Pro Ser Asp Ala Asp Pro Trp Lys Ala Phe Met Ala
            100                 105                 110

CAA GTG GAA GTA CTG ATA GAT AAG AAA ATA GAG GAG TAT GCT AAA AGT       384
Gln Val Glu Val Leu Ile Asp Lys Lys Ile Glu Glu Tyr Ala Lys Ser
        115                 120                 125
```

```
AAA GCT CTT GCA GAG TTA CAG GGT CTT CAA AAT AAT TTT GAA GAT TAT      432
Lys Ala Leu Ala Glu Leu Gln Gly Leu Gln Asn Asn Phe Glu Asp Tyr
    130                 135                 140

GTA AAT GCG TTG GAT TCC TGG AAG AAA GCG CCT GTA AAT TTA CGA AGT      480
Val Asn Ala Leu Asp Ser Trp Lys Lys Ala Pro Val Asn Leu Arg Ser
145                 150                 155                 160

CGA AGA AGC CAA GAT CGA ATA AGA GAA CTT TTT TCT CAA GCA GAA AGC      528
Arg Arg Ser Gln Asp Arg Ile Arg Glu Leu Phe Ser Gln Ala Glu Ser
                165                 170                 175

CAT TTT CGT AAT TCC ATG CCG TCA TTT GCG GTT TCC AAA TTC GAA GTT      576
His Phe Arg Asn Ser Met Pro Ser Phe Ala Val Ser Lys Phe Glu Val
            180                 185                 190

CTG TTT CTA CCA ACA TAT GCA CAA GCT GCA AAT ACA CAT TTA TTG CTA      624
Leu Phe Leu Pro Thr Tyr Ala Gln Ala Ala Asn Thr His Leu Leu Leu
        195                 200                 205

TTA AAA GAT GCT CAA GTT TTT GGA GAA GAA TGG GGA TAT TCT TCA GAA      672
Leu Lys Asp Ala Gln Val Phe Gly Glu Glu Trp Gly Tyr Ser Ser Glu
    210                 215                 220

GAT ATT GCT GAA TTT TAT CAA AGA CAA TTA AAA CTT ACG CAA CAA TAC      720
Asp Ile Ala Glu Phe Tyr Gln Arg Gln Leu Lys Leu Thr Gln Gln Tyr
225                 230                 235                 240

ACT GAC CAT TGT GTC AAT TGG TAT AAT GTT GGA TTA AAT AGT TTA AGA      768
Thr Asp His Cys Val Asn Trp Tyr Asn Val Gly Leu Asn Ser Leu Arg
                245                 250                 255


GGT TCA ACT TAT GAT GCA TGG GTC AAA TTT AAC CGT TTT CGC AGA GAA      816
Gly Ser Thr Tyr Asp Ala Trp Val Lys Phe Asn Arg Phe Arg Arg Glu
            260                 265                 270

ATG ACA TTA ACT GTA TTA GAT CTA ATT GTA TTA TTC CCA TTT TAT GAT      864
Met Thr Leu Thr Val Leu Asp Leu Ile Val Leu Phe Pro Phe Tyr Asp
        275                 280                 285

GTT CGG TTA TAC TCA AAA GGA GTT AAA ACA GAA CTA ACA AGA GAC ATT      912
Val Arg Leu Tyr Ser Lys Gly Val Lys Thr Glu Leu Thr Arg Asp Ile
    290                 295                 300

TTT ACA GAT CCA ATT TTT ACA CTC AAT GCT CTT CAA GAG TAT GGA CCA      960
Phe Thr Asp Pro Ile Phe Thr Leu Asn Ala Leu Gln Glu Tyr Gly Pro
305                 310                 315                 320

ACT TTT TCG AGT ATA GAA AAC TCT ATT CGA AAA CCT CAT TTA TTT GAT     1008
Thr Phe Ser Ser Ile Glu Asn Ser Ile Arg Lys Pro His Leu Phe Asp
                325                 330                 335

TAT TTG CGT GGG ATT GAA TTT CAT ACG CGT CTT CGA CCT GGT TAC TCT     1056
Tyr Leu Arg Gly Ile Glu Phe His Thr Arg Leu Arg Pro Gly Tyr Ser
            340                 345                 350

GGG AAA GAT TCT TTC AAT TAT TGG TCT GGT AAT TAT GTA GAA ACT AGA     1104
Gly Lys Asp Ser Phe Asn Tyr Trp Ser Gly Asn Tyr Val Glu Thr Arg
        355                 360                 365

CCT AGT ATA GGA TCT AAT GAT ACA ATC ACT TCC CCA TTT TAT GGA GAT     1152
Pro Ser Ile Gly Ser Asn Asp Thr Ile Thr Ser Pro Phe Tyr Gly Asp
    370                 375                 380
```

```
AAA TCT ATT GAA CCT ATA CAA AAG CTA AGC TTT GAT GGA CAA AAA GTT      1200
Lys Ser Ile Glu Pro Ile Gln Lys Leu Ser Phe Asp Gly Gln Lys Val
385                 390                 395                 400

TAT CGA ACT ATA GCT AAT ACA GAC ATA GCG GCT TTT CCG GAT GGC AAG      1248
Tyr Arg Thr Ile Ala Asn Thr Asp Ile Ala Ala Phe Pro Asp Gly Lys
                405                 410                 415

ATA TAT TTT GGT GTT ACG AAA GTT GAT TTT AGT CAA TAT GAT GAT CAA      1296
Il  Tyr Phe Gly Val Thr Lys Val Asp Phe Ser Gln Tyr Asp Asp Gln
            420                 425                 430

AAA AAT GAA ACT AGT ACA CAA ACA TAT GAT TCA AAA AGA TAC AAT GGC      1344
Lys Asn Glu Thr Ser Thr Gln Thr Tyr Asp Ser Lys Arg Tyr Asn Gly
        435                 440                 445

TAT TTA GGT GCA CAG GAT TCT ATC GAC CAA TTA CCA CCA GAA ACA ACA      1392
Tyr Leu Gly Ala Gln Asp Ser Ile Asp Gln Leu Pro Pro Glu Thr Thr
    450                 455                 460

GAT GAA CCA CTT GAA AAA GCA TAT AGT CAT CAG CTT AAT TAC GCA GAA      1440
Asp Glu Pro Leu Glu Lys Ala Tyr Ser His Gln Leu Asn Tyr Ala Glu
465                 470                 475                 480

TGT TTC TTA ATG CAG GAC CGT CGT GGA ACA ATT CCA TTT TTT ACT TGG      1488
Cys Phe Leu Met Gln Asp Arg Arg Gly Thr Ile Pro Phe Phe Thr Trp
                485                 490                 495

ACA CAT AGA AGT GTA GAC TTT TTT AAT ACA ATT GAT GCT GAA AAA ATT      1536
Thr His Arg Ser Val Asp Phe Phe Asn Thr Ile Asp Ala Glu Lys Ile
            500                 505                 510

ACT CAA CTT CCA GTA GTG AAA GCA TAT GCC TTG TCT TCA GGC GCT TCC      1584
Thr Gln Leu Pro Val Val Lys Ala Tyr Ala Leu Ser Ser Gly Ala Ser
        515                 520                 525

ATT ATT GAA GGT CCA GGA TTC ACA GGA GGA AAT TTA CTA TTC CTA AAA      1632
Ile Ile Glu Gly Pro Gly Phe Thr Gly Gly Asn Leu Leu Phe Leu Lys
    530                 535                 540

GAA TCT AGT AAT TCA ATT GCT AAA TTT AAA GTT ACC TTA AAT TCA GCA      1680
Glu Ser Ser Asn Ser Ile Ala Lys Phe Lys Val Thr Leu Asn Ser Ala
545                 550                 555                 560

GCC TTG TTA CAA CGA TAT CGC GTA AGA ATA CGC TAT GCT TCA ACC ACT      1728
Ala Leu Leu Gln Arg Tyr Arg Val Arg Ile Arg Tyr Ala Ser Thr Thr
                565                 570                 575

AAC CTA CGA CTT TTC GTG CAA AAT TCA AAC AAT GAT TTT CTT GTC ATC      1776
Asn Leu Arg Leu Phe Val Gln Asn Ser Asn Asn Asp Phe Leu Val Ile
            580                 585                 590

TAC ATT AAT AAA ACT ATG AAT ATA GAT GGT GAT TTA ACA TAT CAA ACA      1824
Tyr Ile Asn Lys Thr Met Asn Ile Asp Gly Asp Leu Thr Tyr Gln Thr
        595                 600                 605

TTT GAT TTC GCA ACT AGT AAT TCT AAT ATG GGA TTC TCT GGT GAT ACA      1872
Phe Asp Phe Ala Thr Ser Asn Ser Asn Met Gly Phe Ser Gly Asp Thr
    610                 615                 620

AAT GAC TTT ATA ATA GGA GCA GAA TCT TTC GTT TCT AAT GAA AAA ATC      1920
Asn Asp Phe Ile Ile Gly Ala Glu Ser Phe Val Ser Asn Glu Lys Ile
625                 630                 635                 640

TAT ATA GAT AAG ATA GAA TTT ATC CCA GTA CAA                         1953
Tyr Ile Asp Lys Ile Glu Phe Ile Pro Val Gln
                645                 650
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 651 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus thuringiensis
(B) STRAIN: tolworthi
(C) INDIVIDUAL ISOLATE: 43F

(vii) IMMEDIATE SOURCE:
(B) CLONE: E. coli XL1-Blue (pM1,98-4), NRRL B-18291

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..651

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Asn Pro Asn Asn Arg Ser Glu Tyr Asp Thr Ile Lys Val Thr Pro
1               5                  10                  15

Asn Ser Glu Leu Pro Thr Asn His Asn Gln Tyr Pro Leu Ala Asp Asn
            20                  25                  30

Pro Asn Ser Thr Leu Glu Glu Leu Asn Tyr Lys Glu Phe Leu Arg Met
        35                  40                  45

Thr Ala Asp Asn Ser Thr Glu Val Leu Asp Ser Ser Thr Val Lys Asp
    50                  55                  60

Ala Val Gly Thr Gly Ile Ser Val Val Gly Gln Ile Leu Gly Val Val
65                  70                  75                  80

Gly Val Pro Phe Ala Gly Ala Leu Thr Ser Phe Tyr Gln Ser Phe Leu
                85                  90                  95

Asn Ala Ile Trp Pro Ser Asp Ala Asp Pro Trp Lys Ala Phe Met Ala
            100                 105                 110

Gln Val Glu Val Leu Ile Asp Lys Lys Ile Glu Glu Tyr Ala Lys Ser
        115                 120                 125

Lys Ala Leu Ala Glu Leu Gln Gly Leu Gln Asn Asn Phe Glu Asp Tyr
    130                 135                 140

Val Asn Ala Leu Asp Ser Trp Lys Lys Ala Pro Val Asn Leu Arg Ser
145                 150                 155                 160

Arg Arg Ser Gln Asp Arg Ile Arg Glu Leu Phe Ser Gln Ala Glu Ser
                165                 170                 175

His Phe Arg Asn Ser Met Pro Ser Phe Ala Val Ser Lys Phe Glu Val
            180                 185                 190

Leu Phe Leu Pro Thr Tyr Ala Gln Ala Ala Asn Thr His Leu Leu Leu
        195                 200                 205
```

```
L u Lys Asp Ala Gln Val Ph  Gly Glu Glu Trp Gly Tyr Ser Ser Glu
    210                 215                 220

Asp Ile Ala Glu Phe Tyr Gln Arg Gln Leu Lys Leu Thr Gln Gln Tyr
225                 230                 235                 240

Thr Asp His Cys Val Asn Trp Tyr Asn Val Gly Leu Asn Ser Leu Arg
                245                 250                 255

Gly Ser Thr Tyr Asp Ala Trp Val Lys Phe Asn Arg Phe Arg Arg Glu
            260                 265                 270

Met Thr Leu Thr Val Leu Asp Leu Ile Val Leu Phe Pro Phe Tyr Asp
        275                 280                 285

Val Arg Leu Tyr Ser Lys Gly Val Lys Thr Glu Leu Thr Arg Asp Ile
    290                 295                 300

Phe Thr Asp Pro Ile Phe Thr Leu Asn Ala Leu Gln Glu Tyr Gly Pro
305                 310                 315                 320

Thr Phe Ser Ser Ile Glu Asn Ser Ile Arg Lys Pro His Leu Phe Asp
                325                 330                 335

Tyr Leu Arg Gly Ile Glu Phe His Thr Arg Leu Arg Pro Gly Tyr Ser
            340                 345                 350

Gly Lys Asp Ser Phe Asn Tyr Trp Ser Gly Asn Tyr Val Glu Thr Arg
        355                 360                 365

Pro Ser Ile Gly Ser Asn Asp Thr Ile Thr Ser Pro Phe Tyr Gly Asp
    370                 375                 380

Lys Ser Ile Glu Pro Ile Gln Lys Leu Ser Phe Asp Gly Gln Lys Val
385                 390                 395                 400

Tyr Arg Thr Ile Ala Asn Thr Asp Ile Ala Ala Phe Pro Asp Gly Lys
                405                 410                 415

Ile Tyr Phe Gly Val Thr Lys Val Asp Phe Ser Gln Tyr Asp Asp Gln
            420                 425                 430

Lys Asn Glu Thr Ser Thr Gln Thr Tyr Asp Ser Lys Arg Tyr Asn Gly
        435                 440                 445

Tyr Leu Gly Ala Gln Asp Ser Ile Asp Gln Leu Pro Pro Glu Thr Thr
    450                 455                 460

Asp Glu Pro Leu Glu Lys Ala Tyr Ser His Gln Leu Asn Tyr Ala Glu
465                 470                 475                 480

Cys Phe Leu Met Gln Asp Arg Arg Gly Thr Ile Pro Phe Phe Thr Trp
                485                 490                 495

Thr His Arg Ser Val Asp Phe Phe Asn Thr Ile Asp Ala Glu Lys Ile
            500                 505                 510

Thr Gln Leu Pro Val Val Lys Ala Tyr Ala Leu Ser Ser Gly Ala Ser
        515                 520                 525

Ile Ile Glu Gly Pro Gly Phe Thr Gly Gly Asn Leu Leu Phe Leu Lys
    530                 535                 540

Glu Ser Ser Asn Ser Ile Ala Lys Phe Lys Val Thr Leu Asn Ser Ala
545                 550                 555                 560

Ala Leu Leu Gln Arg Tyr Arg Val Arg Ile Arg Tyr Ala Ser Thr Thr
                565                 570                 575
```

```
Asn Leu Arg Leu Phe Val Gln Asn Ser Asn Asn Asp Phe Leu Val Ile
            580                 585                 590

Tyr Ile Asn Lys Thr Met Asn Ile Asp Gly Asp Leu Thr Tyr Gln Thr
        595                 600                 605

Phe Asp Phe Ala Thr Ser Asn Ser Asn Met Gly Phe Ser Gly Asp Thr
    610                 615                 620

Asn Asp Phe Ile Ile Gly Ala Glu Ser Phe Val Ser Asn Glu Lys Ile
625                 630                 635                 640

Tyr Ile Asp Lys Ile Glu Phe Ile Pro Val Gln
                645                 650
```

Claims

1. A method for controlling scarab pests which comprises contacting said pests with a scarab-controlling effective amount of a *Bacillus thuringiensis* strain or toxin selected from the group consisting of *Bacillus thuringiensis* PS86B1, *Bacillus thuringiensis* PS43F, *Bacillus thuringiensis* PS50C, toxins of said microbes, and variants of said microbes and toxins which have activity against scarabs.

2. The method, according to claim 1, wherein said *Bacillus thuringiensis* is *Bacillus thuringiensis* PS86B1.

3. The method, according to claim 1, wherein said *Bacillus thuringiensis* is *Bacillus thuringiensis* PS43F.

4. The method, according to claim 1, wherein said *Bacillus thuringiensis* is *Bacillus thuringiensis* PS50C.

5. A composition of matter for controlling scarab pests comprising a *Bacillus thuringiensis* strain or toxin selected from the group consisting of *Bacillus thuringiensis* PS86B1, *Bacillus thuringiensis* PS43F, *Bacillus thuringiensis* PS50C, toxins of said microbes, and variants of said microbes and toxins which have activity against scarabs, in association with an inert carrier.

6. A method for controlling scarab pests which comprises contacting said scarab pests with a scarab-controlling amount of a pesticidal composition comprising intact treated cells having prolonged pesticidal activity when applied to the environment of scarab larvae, wherein said insecticide is produced by a gene which encodes a toxin selected from the group consisting of toxins expressed by a *Bacillus thuringiensis* selected from the group consisting of *Bacillus thuringiensis* PS86B1, *Bacillus thuringiensis* PS43F, *Bacillus thuringiensis* PS50C, and toxins which are variants of the toxins expressed by said microbes and which have activity against scarabs.

7. A polynucleotide sequence comprising DNA wherein said DNA encodes a toxin which is active against scarab pests and wherein said DNA, or a variant thereof, is obtainable from a *Bacillus thuringiensis* strain selected from the group consisting of *Bacillus thuringiensis* PS86B1, *Bacillus thuringiensis* PS43F, *Bacillus thuringiensis* PS50C, and variants of said microbes which have activity against scarabs.

8. The polynucleotide sequence, according to claim 7, comprising DNA encoding all or part of SEQ ID NO. 2.

9. The polynucleotide sequence, according to claim 8, comprising DNA having all or part of SEQ ID NO. 1.

10. The polynucleotide sequence, according to claim 7, comprising DNA encoding all or part of SEQ ID NO. 4.

11. The polynucleotide sequence, according to claim 10, comprising DNA having all or part of SEQ ID NO. 3.

12. The polynucleotide sequence, according to claim 7, comprising DNA encoding all or part of SEQ ID NO. 6.

13. The polynucleotide sequence, according to claim 12, comprising DNA having all or part of SEQ ID NO. 5.

14. A toxin which is active against scarab pests and which is encoded by a polynucleotide sequence of claim 7.

15. The toxin, according to claim 14, having all or part of SEQ ID NO. 2.

16. The toxin, according to claim 14, having all or part of SEQ ID NO. 4.

17. The toxin, according to claim 14, having all or part of SEQ ID NO. 6.

18. A plant cell transformed by a polynucleotide sequence of claim 7.

19. A microbe transformed by a polynucleotide sequence of claim 7.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/32, A01N 63/00
C12N 15/82, 1/21, C12P 1/04 //
(C12P 1/04, C12R 1:07)

A3

(11) International Publication Number: WO 93/15206

(43) International Publication Date: 5 August 1993 (05.08.93)

(21) International Application Number: PCT/US93/00966

(22) International Filing Date: 1 February 1993 (01.02.93)

(30) Priority data:
07/828,430 30 January 1992 (30.01.92) US

(71) Applicant: MYCOGEN CORPORATION [US/US]; 4980 Carroll Canyon Road, San Diego, CA 92121 (US).

(72) Inventors: MICHAELS, Tracy, E. ; 1110 Fern Street, Escondido, CA 92027 (US). FONCERRADA, Louis ; 322 Ferrara Way, Vista, CA 92083 (US). NARVA, Kenneth, E. ; 12123 Caminito Mira Del Mar, San Diego, CA 92130 (US).

(74) Agents: SALIWANCHIK, David; R. et al.; Saliwanchik & Saliwanchik, 2421 N.W. 41st Street, Suite A-1, Gainesville, FL 32606 (US).

(81) Designated States: AU, BR, CA, JP, KR, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
6 January 1994 (06.01.94)

(54) Title: PROCESS FOR CONTROLLING SCARAB PESTS WITH *BACILLUS THURINGIENSIS* ISOLATES

(57) Abstract

Certain isolates of *Bacillus thuringiensis* (B.t.) have been found to have activity against scarab pests. These isolates are designated B.t. PS86B1, B.t. PS43F and B.t. PS50C. These isolates, or transformed hosts containing the gene expressing a scarab-active toxin obtained from the isolates, can be used to control scarab-active pests, e.g., masked chafer, *Cyclocephala* sp., June bettle, *Cotinis* sp., northern masked chafer, *Cyclocephala borealis*, Japanese beetle, *Popillia japonica*, and Pasadena masked chafer, *Cyclocephala pasadenae*, in various environments.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic			SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	UA	Ukraine
DE	Germany	MG	Madagascar	US	United States of America
DK	Denmark	ML	Mali	UZ	Uzbekistan
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/00966

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/32 A 01 N 63/00 C 12 N 15/82
C 12 N 1/21 C 12 P 1/04 //(C 12 P 1/04 C 12 R 1:07)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K C 12 N A 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X Y	EP,A,0330342 (MYCOGEN CORPORATION) 30 August 1989 see the whole document	1-2,5-6 7,14,18 -19
Y	MICROBIOLOGICAL REVIEWS vol. 53, no. 2, June 1989, WASHINGTON DC, US pages 242 - 255 H\FTE, H. & WHITELEY, H.R. 'Insecticidal crystal proteins of Bacillus thuringiensis' see the whole document	7,14,18 -19

-/-

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
31-08-1993	03. 12. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	S.M. ANDRES

Form PCT/ISA/210 (second sheet) (January 1985)

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/00966

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/32 A 01 N 63/00 C 12 N 15/82
C 12 N 1/21 C 12 P 1/04 //(C 12 P 1/04 C 12 R 1:07)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K C 12 N A 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X Y	EP,A,0330342 (MYCOGEN CORPORATION) 30 August 1989 see the whole document	1-2,5-6 7,14,18 -19

Y	MICROBIOLOGICAL REVIEWS vol. 53, no. 2, June 1989, WASHINGTON DC, US pages 242 - 255 H\FTE, H. & WHITELEY, H.R. 'Insecticidal crystal proteins of Bacillus thuringiensis' see the whole document	7,14,18 -19
	--- -/-	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
31-08-1993	03. 12. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	S.M. ANDRES

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF INVERTEBRATE PATHOLOGY vol. 27, no. 3, May 1976, NEW YORK, US pages 421 - 422 SHARPE, E.S. 'Toxicity of the parasporal crystal of Bacillus thuringiensis to japanese beetle larvae' see the whole document ---	1,6
A	LETTERS IN APPLIED MICROBIOLOGY vol. 14, no. 2, 29 January 1992, OXFORD, GB pages 54 - 57 OHBA, M. ET AL. 'A unique isolate of Bacillus thuringiensis serovar japonensis with high larvicidal activity specific for scarabaeid beetles' see the whole document ---	1,6
A	THE MADRAS AGRICULTURAL JOURNAL vol. 58, 1971, pages 114 - 116 SHINDE, V. & SHARMA, S. 'Bacteria pathogenic to Lachnosterna consanguinea Blanch (Coleoptera:Scarabaeidae)' see the whole document -----	1,6

INTERNATIONAL SEARCH REPORT

:rnational application No.
PCT/US 93/00966

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see PCT/ISA/206 mailed on 15.09.93

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 5-7, 14, 18-19 (partially); 2 completely

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9300966 SA 70085

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 23/11/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0330342	30-08-89	US-A- 4966765	30-10-90
		JP-A- 2288814	28-11-90
		US-A- 5100665	31-03-92

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82